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Pyrrolidone carboxylic acid levels or caspase-14 expression in the corneocytes of lesional skin correlates with clinical severity, skin barrier function and lesional inflammation in atopic dermatitis

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ABSTRACT

Background: Dry skin in atopic dermatitis (AD) mainly results from barrier impairment due to deficiency of ceramide and natural moisturizing factors including pyrrolidone carboxylic acid (PCA) in stratum corneum (SC). Caspase-14 cleaves filaggrin monomers to free amino acids and their derivatives such as PCA, contributing natural moisturizing factors. Cytokines in the corneocytes represent cutaneous inflammation severity of AD patients.
Object: To analyze the correlations of PCA, caspase-14 and cytokines in corneocytes with clinical severity, barrier function and skin inflammation, those were quantitated.
Methods: A total of 73 persons were enrolled: 21 patients with mild AD, 21 with moderate-to-severe AD, 13 with X-linked ichthyosis (XLI) as a negative control for filaggrin gene (*FLG*) mutation, and 18 healthy controls. Skin barrier functions such as basal transepidermal water loss (TEWL), stratum corneum (SC) hydration and skin surface pH were measured. To collect corneocytes, stripping with D-squame® discs was done on lesional and non-lesional skin. And then PCA was isolated from D-squame® discs and quantitated by LC–MS/MS. Cytokine assays were performed.
Results: The quantity of PCA and caspase-14 was decreased in inflammatory lesions compared to non-lesion in AD patients. And the amounts of PCA and caspase-14 in the lesion of AD patients correlated with clinical severity as determined by eczema area and severity index score and the skin barrier functions. Also, the expressions of TNF-α and IL-13 inversely correlated with PCA quantity.
Conclusion: The quantity of PCA or caspase-14 in the corneocytes of the lesional skin of AD patients reflects the clinical severity, skin barrier function and the degree of lesional inflammation.

1. Introduction

Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease characterized by severe pruritus and xerotic skin. One of the key features of AD is a defective skin barrier. Recently, the loss-of-function mutation of filaggrin gene (*FLG*) was reported as a major predisposing factor for defective skin barrier in AD [1–4].

Filaggrin is a structural protein that is involved in corneocyte development by aggregating keratin filaments in keratinocytes [5]. The *FLG* is located on chromosome 1q21 within the epidermal differentiation complex, and encodes profilaggrin [6]. Profilaggrin is comprised of 10–12 tandem arranged filaggrin repeats, and each repeat contains a short linker region [7]. In the stratum granulosum, translated profilaggrin moves up to the lower layer of the stratum corneum (SC), where it is cleaved into filaggrin monomers which aggregate keratin fibrils [8]. Then, filaggrin monomers are subsequently processed into free amino acids and their derivatives by peptidylarginine deiminase (PAD) 1, PAD 3, caspase-14, calpain 1 and bleomycin hydrolase (BH) [8]. Among them, caspase-14 can directly cleave the filaggrin monomer

Abbreviations: AD, atopic dermatitis; PCA, pyrrolidone carboxylic acid; NMF, natural moisturizing factor; SC, stratum corneum; TEWL, transepidermal water loss; FLG, filaggrin gene; XLI, X-linked ichthyosis.

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probably in preparation for complete breakdown by other proteases [9]. Caspase-14 deficiency leads to the accumulation of fragments derived from filaggrin monomer, suggested by that filaggrin degradation is defective in their caspase-14 deficient mice [10].

Breakdown of filaggrin into hygroscopic free amino acids and their derivatives such as pyrrolidone carboxylic acid (PCA) is the major contributor to the natural moisturizing factor (NMF) [11]. NMFs are important to the function of the SC as they provide moisture retention (humectant), maintain acidic pH and buffering capacity of the SC, promote proper epidermal maturation and desquamation, and decrease pathogenic bacterial colonization [12]. In addition, the ionic interaction between NMF and keratins increases the elastic properties of the SC [13].

Individual carriers of *FLG* mutation with a history of AD had significantly reduced levels of NMF in the SC [14]. Filaggrin deficiency has been associated with many clinical features of AD skin [14,15]. However, *FLG* mutation is not sufficient to explain the increasing prevalence of AD in Korea, because relatively low frequency of *FLG* mutation has been observed in AD patients in Korea. Recent study showed that *FLG* mutations were found in only 71(6.45%) in 1100 Korean AD patients [16]. Among them, pK4022X and c3321delA mutations were most common, which were observed in 50 and 18 AD patients, respectively [16]. This implies that Koreans have a lower likelihood of AD due to *FLG* mutation than other races [17].

Because AD patients without *FLG* mutations also have a defective epidermal barrier [18], there must be additional mechanisms impairing barrier integrity. In addition, many of the available data concern the relationship between *FLG* mutations and clinical characteristics of AD, but few studies have evaluated the expression and processing of PCA in the lesional skin of AD patients without *FLG* mutations. Therefore, this study aimed to quantify PCA and caspase-14 in the corneocytes in a non-invasive manner and then analyze the correlation between the levels of PCA or caspase-14 and the clinical severity, skin barrier functions and skin inflammation in AD patients.

2. Materials and methods

2.1. Clinical investigation

In this study, 73 subjects were recruited. There were 42 AD patients, 18 non-atopic healthy controls and 13 X-linked ichthyosis (XLI) patients. The 18 healthy volunteers were matched by sex and age with AD patients, and the 13 XLI patients were recruited as a negative control for *FLG* mutation. To definitively confirm XLI, fluorescence *in situ* hybridization (FISH) analysis was employed to confirm the deletion of the steroid sulfatase gene (*STS*). We recorded their past and family histories of AD, allergic rhinitis (AR), allergic conjunctivitis (AC) and asthma. The clinical severity of AD was evaluated by eczema area and severity index (EASI) score. EASI score is an index of overall eczema with quantification of the erythema, papulation, excoriation and lichenification of each part of the body [19]. Basal transepidermal water loss (TEWL), SC hydration and skin surface pH were measured to evaluate skin barrier function. Basal TEWL was measured with the Tewameter TM210 (Courage and Khazaka, Cologne, Germany), SC hydration was quantitated as capacitance with the Corneometer CM820 (Courage and Khazaka) and surface pH was measured with a flat glass surfaced electrode attached to a pH meter (WTW, Weilheim, Germany) on lesional and non-lesional skin.

To obtain corneocytes in a non-invasive manner, 20 sheets of D-squame® discs (CuDerm, Dallas, TX, USA) as adhesive tape discs, were stripped in series from both lesional and non-lesional skin. Each tape disc was pressed on the volar aspect of the forearm for 10 s with standardized force using a disc pressure applicator (CuDerm). The acquired D-squame® discs were stored in a 20 ml glass bottle in a −70 °C deep freezer until analysis. In addition, blood sampling was conducted for DNA analysis. Blood was collected in heparin tubes and centrifuged for 5 min at 2500 rpm to separate plasma from blood. The pelleted blood cell samples were stored in a −70 °C deep freezer.

This study was approved by the Institutional Review Board of Yonsei University Wonju College of Medicine (Approval no. 2011-84).

2.2. Quantification of pyrrolidone carboxylic acid (PCA) by liquid chromatography tandem mass spectrometry (LC/MS–MS) analysis

PCA was extracted and quantified from three sheets of D-squame® discs (CuDerm) containing the corneocytes stripped from the skin surface. To measure the extracted PCA, liquid chromatography with tandem mass spectrometry (LC/MS–MS) analysis was employed. Before LC/MS–MS analysis, 1 ml of 100% ethanol and 50 μg of L-homoserine as an internal standard (IS) were added to the tape strips, followed by 1 h of continuous shaking. After the tape was partially dissolved by ethanol, the corneocytes were gathered by cell scraper and placed in 1.5 ml microcentrifuge tubes. These were shaken for one additional hour and centrifuged at 12,000 rpm for 10 min. The upper layer of liquid was moved into a new vial and 5 μl was injected into the Alliance HT (Waters, Milford, MA, USA) for LC/MS–MS analysis.

A SUPELCOSIL™ LC-18-DB LC column (Sigma-Aldrich, St. Louis, MO, USA) was used with the mobile phase consisting of buffer A (0.1% formic acid in methanol) and buffer B (0.1% formic acid in deionized water). A portion was injected at 0.45 ml/min into the LC system. The column was re-equilibrated at initial conditions for 10 min. MS–MS analysis was carried out on a Quattro Premier XE (Waters) with electrospray ionization in the positive ion mode. The following parameters were found to give the best sensitivity: capillary temperature = 120 °C; capillary voltage = 4 kV; cone voltage = 25 V (PCA) or 10 V (IS); collision energy = 15 V (PCA) or 10 V (IS); source and desolvation temperatures = 120 °C and 280 °C, respectively; source and desolvation gas flow = 500 L/h and 50 L/h, respectively.

PCA concentration was calculated by plotting nominal concentrations versus response factors of analyte (peak area of PCA/peak area of IS). A quadratic fit gave the best results. LC/MS–MS program was carried out with Masslynx Software version 4.1 (Waters). PCA quantity was calculated as PCA concentration per total protein.

2.3. Quantification of caspase-14 expression by enzyme-linked immunosorbent assay (ELISA) analysis

Caspase-14 is an enzyme that degrades filaggrin monomers to free amino acids and their derivatives including PCA in corneocytes. Caspase-14 was measured by ELISA and quantified in nanograms of protein. These data were compared among AD patients, XLI patients (the negative control group for *FLG* mutation) and normal healthy controls. Protein samples were extracted from the corneocytes on three D-squame® discs (CuDerm) using the Qproteome Mammalian Protein Prep kit (Qiagen, Hilden, Germany). The concentration of corneocyte proteins on the disc tape was determined by Bradford assay. The expression level of caspase-14 in the corneocytes was determined with a human caspase-14 ELISA kit (USCN Life Science Inc., Wuhan, China). Samples and standards were added to plates precoated with an antibody specific to caspase-14 and incubated at 37 °C for 2 h. Avidin conjugated to horseradish peroxidase was then added to