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Reduced humidity induces skin barrier dysfunction and secretion of dipeptidyl peptidase-4 (DPP-4) in a skin-equivalent model

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Abstract: Seasonal changes can affect the physiological condition of the skin and cause various cutaneous disorders. The skin barrier function tends to worsen during winter when humidity is lower compared to other seasons. To determine the influence of relative humidity (RH) on the function of the skin barrier, we performed biological and histological assays using skin equivalents that were cultured under reduced humidity in an environmental humidity chamber. We found that reduced humidity led to decreased epidermal thickness and disruption of the skin barrier. Reduced humidity induced the decrease of filaggrin, loricrin and damage to tight junction. In addition, dipeptidyl peptidase-4 (DPP4), which has roles in the immunological process, was upregulated in a skin-equivalent model under reduced humidity. These results suggest that reduced humidity affects the skin barrier function and regulates the secretion of DPP4 in a skin-equivalent model.

Keywords: humidity; skin equivalent; barrier function; DPP4

INTRODUCTION

Seasonal changes affect the condition of normal skin and may also cause various cutaneous disorders. These skin disorders tend to worsen during winter when humidity is low [1,2]. A substantial decrease in environmental humidity decreases the water content by reducing total free amino acid generation [3]. The water content of the skin is also regulated by skin barrier functions, which depend on lipid lamellar structure [4], tight junctions [5] and on natural moisturizing factors [6]. The skin barrier functions deteriorate due to ultraviolet radiation, low humidity and psychological stress [7]. In particular, low humidity may trigger skin disorders and tends to worsen conditions such as atopic dermatitis and psoriasis [1,2]. Changes in environmental humidity influence the release of proteins from skin. Low humidity increases IL-1α in the epidermis [8] and histamine in the dermis [9]. In an epidermal organotypic culture system, low humidity induces synthesis and release of cortisol [10].

Dipeptidyl peptidase-4 (DPP4 or CD26) is a 110-kDa membrane-anchored glycoprotein with a variety of functions. It has roles in the immune response, and abnormal expression is found in autoimmune disease [11]. The keratinocytes of normal human skin express DPP4 sporadically, whereas increased expression of DPP4 was observed in inflammatory skin diseases such as psoriasis and spongiotic dermatitis in the basal and spinous layers [12-14]. However, little data are available concerning the effect of environmental humidity change on DPP4 regulation.

In the present study, we examined the effects of changes in environmental humidity in a skin-equivalent model. We cultured a skin-equivalent model under standard *in vitro* humidity (80% RH) and reduced humidity (60% RH) for 48 h and evaluated the skin barrier function and change in DPP4 protein level.

MATERIALS AND METHODS

Culture of skin-equivalent model

Skin equivalents (SEs), EpiDerm™ FT-400 (Mat-Tek Corp, Ashland, MA, USA) were removed from agarose-containing 6-well plates and moved to 6-well plates filled with EpiDerm™ Full Thickness medium. SEs were cultured at 37°C, 5% CO_2, for 48 h and then transferred to an environmental humidity chamber. SEs were cultured for 48 h at reduced humidity (60% RH) and standard *in vitro* humidity (80% RH).

Tight junction (TJ) permeability assay

The TJ permeability assay was carried out to evaluate TJ functions using a surface biotinylation technique [15]. In brief, SEs were incubated with 2 mg/mL EZ Link sulfo NHS LC Biotin (Pierce, Rockford, IL, USA) in phosphate-buffered saline (PBS) containing 1 mM $CaCl_2$ on the dermal side. After 30 min of incubation, SEs were separated and frozen in the OCT optimal cutting temperature (OCT) compound. Frozen sections of 10 μm thickness were air-dried overnight and soaked for 1 h in 1% bovine serum albumin (BSA)/PBS. The samples were incubated for 1 h with a streptavidin Texas Red (Calbiochem, Darmstadt, Germany). Microscopy was performed on an LSM 510 confocal microscope (Carl Zeiss, Germany).

Quantitative real-time PCR analysis

Total RNA from the epidermal part of the SE was isolated using TRIzol™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two-microgram RNA samples were reverse-transcribed into cDNA using SuperScript®III reverse transcriptase (Invitrogen), and aliquots were stored at -20°C. The cDNA samples were analyzed for filaggrin (Hs00856927_g1) and loricrin (Hs01894962_s1); the mRNA expression levels were normalized to the expression of GAPDH. Quantitative real-time TaqMan RT-PCR (Applied Biosystems, Foster City, CA, USA) was used to determine the expression levels of selected target genes. The cycling conditions were as follows: denaturing step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. Quantitation of gene expression was carried out by the comparative C_t method. All data were obtained from more than two independent experiments carried out in triplicate.

Western blotting

The epidermal parts of SEs were lysed in cell lysis buffer containing protease inhibitors. The lysates were then centrifuged at 15000×*g* for 10 min and the supernatants were used for Western blot analysis. Protein concentrations were determined by the bicinchoninic acid assay (BCA) method using BSA as a standard. The lysates (20 μg protein per well) were fractionated by sodium dodecyl sulfate (SDS) polyacrylamide-gel electrophoresis -(PAGE) and transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.15% Tween-20) for 1 h at room temperature and subsequently probed overnight at 4°C with anti-filaggrin antibody (Novocastra, Newcastle, UK), anti-loricrin antibody (Abcam, Cambridge, UK) and anti-β-actin (Santa Cruz, Dallas, TX, USA). Blots were washed three times with TBST and incubated with horseradish peroxidase-conjugated IgG (Invitrogen, Carlsbad, CA, USA) at room temperature for 1 h. Detection was performed with the ECL system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Immunofluorescence microscopy

Immunofluorescence microscopy was carried out using the following antibodies: anti-filaggrin monoclonal antibody (abcam, Cambridge, UK) and anti-loricrin polyclonal antibody (Abcam). Frozen sections of 10 μm thickness were air dried overnight and soaked in 1% BSA/phosphate-buffered saline for 1 h. The samples were then incubated with primary antibodies for 2 h, washed three times with PBS and incubated with a mixture of fluorescence conjugated secondary antibodies. Microscopy was performed on an LSM 510 confocal microscope (Carl Zeiss, Germany). Laser excitation wavelengths included 405, 488, and 543 nm in sequence by the line method.

Analysis of protein secretion

After 48 h of cultivation at 60% RH, the culture medium was used in a protein array, performed according

to the protocol of the Proteome Profiler Array Human XL cytokine array kit (R&D, Minneapolis, MN, USA) as provided by the manufacturer.

Histology

SEs were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned. Hematoxylin and eosin (H&E) staining was performed. DPP4 was detected in paraffin-embedded sections of SEs using Human DPP4 antibody (R&D, Minneapolis, MN, USA) followed by secondary antibody incubation.

Statistical analysis

Data are expressed as the mean±standard deviation (SD), and the two-tailed Student's *t*-test was used for statistical comparisons. A value of $p<0.05$ was considered statistically significant (indicated by $^{*}p < 0.05$, $^{**}p < 0.01$).

RESULTS

Reduced humidity damaged skin barrier

Initially SEs were cultured at different RH to determine the condition for reduced humidity. After 48 h at 80% RH, which tends to be the standard of RH in a normal tissue culture incubator, the SEs were transferred to environmental humidity chambers at 40% and 60% RH for 4 days or kept at 80% RH as a control. A drop in RH from 80% to 40% seriously altered the morphology of the SEs, but SEs cultured at 60% RH retained normal tissue structure (Supplementary Fig. S1). Even though it is known that 60% RH is not representative of a dry condition for human skin, we set 60% RH as the reduced humidity condition because of the limitations observed *in vitro*. Thus, we compared SEs cultured at 80% RH and 60% RH in subsequent experiments. SEs cultured at 60% RH for 48 h had decreased epidermal thickness, especially of the granular layer (Fig. 1A). We also used the TJ intercellular permeability assay to evaluate the barrier functions of the SEs. At 60% RH, the tracer passed through the stratum corneum of the SEs (Fig. 1B). We found that TJ structure was damaged at reduced humidity.

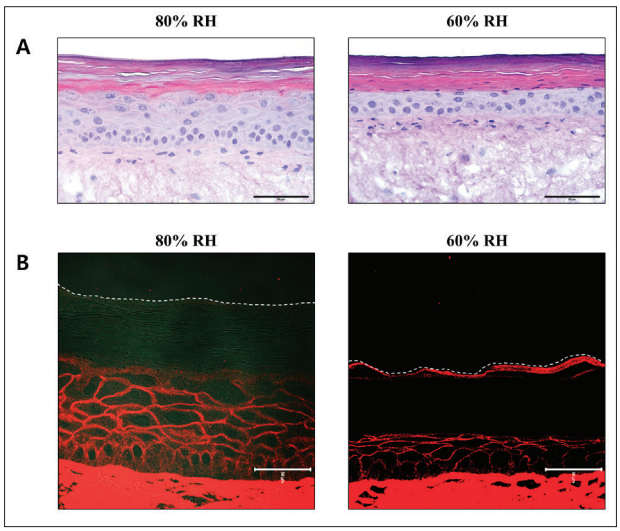

Fig. 1. Reduced humidity impairs the skin barrier. SEs were incubated in an environmental humidity chamber at 80% and 60% RH for 48 h. **A** – SEs cultured at 60% RH for 48 h had decreased epidermal thickness, especially of the granular layer. **B** – The TJ permeability assay was performed to investigate the effect of reduced humidity on TJ structure. In SEs cultured at 80% RH, the diffusion of the tracer was blocked at the stratum corneum. The tracer was capable of passing through the stratum corneum in SEs cultured at 60% RH. The white lines on the image represent the stratum corneum. Scale bar: 50 μm.

Reduced humidity decreased the expression of filaggrin and loricrin.

Next, we analyzed the gene expression related to skin barrier function under reduced humidity. We observed decreased expression of the genes related to terminal differentiation of the skin, filaggrin and loricrin (Fig. 2A). In addition, the levels of respective proteins corresponding to these genes were decreased at 60% RH, as detected by Western blotting (Fig. 2B). Likewise, immunofluorescence staining with anti-filaggrin and anti-loricrin revealed that their patterns were also altered, and the positive-staining layer was decreased at 60% RH (Fig. 2C). These findings indicate that reduced humidity damaged the epidermal structure of SEs.

Reduced humidity induced secretion of DPP4 in a skin-equivalent model.

We assessed the influence of reduced humidity on SEs by using a kit that measures a panel of secreted cytokines, chemokines, growth factors and other

soluble proteins. The change in humidity influenced the secretion of proteins (Fig. 3A). According to the array, we identified DPP4, extracellular matrix metalloproteinase inducer (EMMPRIN), growth/differentiation factor 15 (GDF-15) and hepatocyte growth factor (HGF) at elevated concentrations at reduced humidity. Secreted proteins with decreased abundance at reduced humidity were urokinase receptor (uPAR), macrophage inflammatory protein 3α (MIP-3α), cardiac biomarker ST2, a member of the interleukin-1 receptor family biomarker, and leptin. Among the secreted proteins that were statistically different, DPP4 was the one with the highest difference, being at least three-times higher at 60% RH when compared to 80% RH (Fig. 3B). Because of the high amount of DPP4 released in the media, it was postulated that cells under reduced RH also had an increased amount of this enzyme in their tissues. Similarly to the secretome data presented, the amount of DPP4 was found to be higher when the SEs were analyzed by ImmunoHistoChemistry (IHC), and this difference was pronounced in the suprabasal layer of the SEs (Fig. 3C).

DISCUSSION

Changes in humidity can influence the physiological condition of the skin and cause various cutaneous disorders. A previous study showed that immunohistochemical staining of filaggrin became faint in the epidermis of mice transferred from a humid or normal to a dry environment [3]. Acute disruption of the skin barrier resulted in decreased expression of filaggrin and loricrin. However, prolonged exposure to a low (<10% RH) humidity increased profilaggrin and loricrin [15].

Exposure to low humidity can alter protein secretion in the skin. A previous report pointed to upregulation of the cutaneous immune reaction at low humidity [16]. An immunohistochemical study showed that animals kept in a low humidity environment had greater levels of epidermal IL-1α [8]. Research indicated that cultured epidermal equivalents had increased secretion of IL-1α and IL-8 at low humidity [7]. In our experiment, the low signal intensity made it difficult to compare the secretion of IL-1α and IL-8 at reduced humidity.

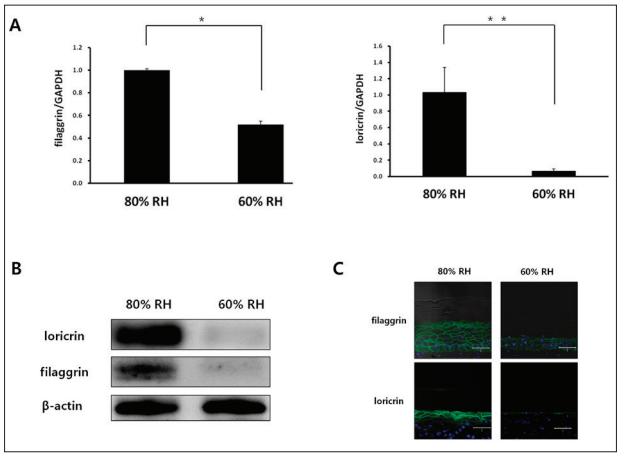

Fig. 2. Reduced humidity decreased the expression of filaggrin and loricrin. The epidermal layers of SEs were harvested to investigate the expression levels of filaggrin and loricrin. **A** – The expression of filaggrin and loricrin mRNA was decreased at 60% RH. **B** – The expression levels of filaggrin and loricrin protein measured by Western blotting. **C** – OCT-embedded sections were immunohistochemically stained; 60% RH decreased the staining of filaggrin and loricrin. Values represent the means±SD of three independent experiments (*p<0.05, **p<0.01).

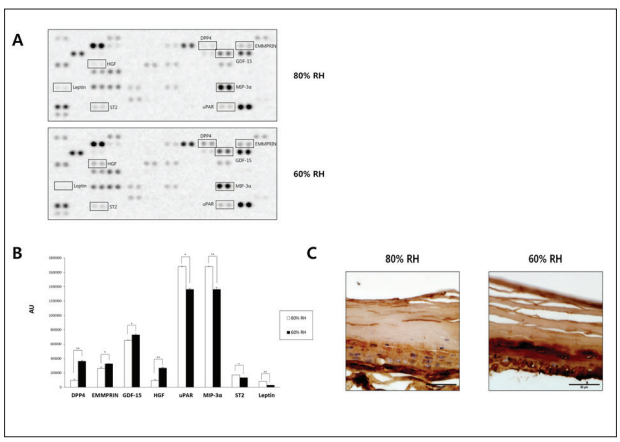

Fig. 3. Reduced humidity changes the secretome profile and DPP4 abundance in an SE model. **A** – Secreted proteins were measured by the cytokine array kit. **B** – The analysis of secreted proteins in SEs cultured at 60% RH. **C** – Paraffin-embedded sections were immunohistochemically stained to analyze the DPP4 staining. Scale bar: 50 μm. (**p<0.01).

In a human organotypic skin model, temperature and humidity change affected epidermal differentiation and led to matrix remodeling [17]. The conditioned medium from an epidermal model cultured at low humidity enhanced MMP-1 secretion by normal human dermal fibroblasts [7]. DPP4, EMMPRIN, GDF-15, HGF, uPAR, MIP-3α, ST2 and leptin, which

were regulated at reduced humidity in our experiment, are known to have functions in skin remodeling and inflammation [18-24].

Among the secreted proteins, DPP4 has roles in immune responses, and elevated DPP4 occurs in inflammatory skin disease such as psoriasis and atopic dermatitis [14,25]. We identified increased expression of DPP4 in an SE model at reduced humidity conditions. Therefore, a role of DPP4 in the immunological process and skin barrier function at reduced humidity condition can be speculated.

Many cytokine-targeted therapies are used to treat inflammatory skin diseases [26]. It would be interesting to investigate the effects of such anticytokine therapies under low humidity in a SE model. It was reported that tissue expression of DPP4 in an immunological skin response can either be beneficial or aggravating, depending on a possible Th1/Th2 shift [27]. It is worth considering the noxious effects of DPP4 inhibitors in patients with type 2 diabetes mellitus, especially if the patients are suffering from concurrent allergic diseases.

One limitation of our experiment with an SE model is that it did not include immune cells. An SE model with immune cells would be better for examining the effect of humidity and immune responses in light of the many roles of DPP4. With regard to a cosmetic formulation, applying a formula onto the skin cultured at reduced humidity would more closely mimic the *in vivo* situation and could be a good model system for testing the cosmetic product, either in terms of efficacy or security.

In conclusion, this study demonstrated that reduced humidity affects skin barrier function and regulates the secretion of several cytokines, and particularly DPP4, in a skin-equivalent model.

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Author contributions: SH Lee, IH Bae, PA Marinho, CS Lee, and JS Lee designed the study. SH Lee and IH Bae performed most of the experiments. PA Marinho, CS Lee and JS Lee analyzed the data and contributed to the writing of the manuscript.

Conflict of interest disclosure: The authors have no conflicts of interest to declare.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Lee%20et%20al_4286_Supplementary%20Material.pdf