Coal tar induces AHR-dependent skin barrier repair in atopic dermatitis

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Introduction

Atopic dermatitis (AD) is a common inflammatory skin disease that has both genetic and environmental factors in its etiology. The pathogenesis is thought to be driven by cells of the adaptive immune system, mainly T helper 2 (Th2) lymphocytes. Cytokines derived thereof, such as IL-4, IL-5, and IL-13 contribute to disease symptoms in a STAT6-dependent fashion. Using organotypic skin models with primary keratinocytes from AD patients and controls, we found that coal tar activated the aryl hydrocarbon receptor (AHR), resulting in induction of epidermal differentiation. AHR knockdown by siRNA completely abrogated this effect. Coal tar restored filaggrin expression in FLG-haploinsufficient keratinocytes to wild-type levels, and counteracted Th2 cytokine-mediated downregulation of skin barrier proteins. In AD patients, coal tar completely restored expression of major skin barrier proteins, including filaggrin. Using organotypic skin models stimulated with Th2 cytokines IL-4 and IL-13, we found coal tar to diminish spongiosis, apoptosis, and CCL26 expression, all AD hallmarks. Coal tar interfered with Th2 cytokine signaling via dephosphorylation of STAT6, most likely due to AHR-regulated activation of the NRF2 antioxidative stress pathway. The therapeutic effect of AHR activation herein described opens a new avenue to reconsider AHR as a pharmacological target and could lead to the development of mechanism-based drugs for AD.

Topical application of coal tar is one of the oldest therapies for atopic dermatitis (AD), a T helper 2 (Th2) lymphocyte–mediated skin disease associated with loss-of-function mutations in the skin barrier gene, filaggrin (FLG). Despite its longstanding clinical use and efficacy, the molecular mechanism of coal tar therapy is unknown. Using organotypic skin models with primary keratinocytes from AD patients and controls, we found that coal tar activated the aryl hydrocarbon receptor (AHR), resulting in induction of epidermal differentiation. AHR knockdown by siRNA completely abrogated this effect. Coal tar restored filaggrin expression in FLG-haploinsufficient keratinocytes to wild-type levels, and counteracted Th2 cytokine-mediated downregulation of skin barrier proteins. In AD patients, coal tar completely restored expression of major skin barrier proteins, including filaggrin. Using organotypic skin models stimulated with Th2 cytokines IL-4 and IL-13, we found coal tar to diminish spongiosis, apoptosis, and CCL26 expression, all AD hallmarks. Coal tar interfered with Th2 cytokine signaling via dephosphorylation of STAT6, most likely due to AHR-regulated activation of the NRF2 antioxidative stress pathway. The therapeutic effect of AHR activation herein described opens a new avenue to reconsider AHR as a pharmacological target and could lead to the development of mechanism-based drugs for AD.

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Using submerged cultured human keratinocytes and human organotypic skin models from AD patients and healthy volunteers, we studied the effects of coal tar exposure on the epidermal aspects of AD. Our results show that coal tar activates the AHR signaling pathway, resulting in enhanced epidermal differentiation, increased levels of filaggrin, and inhibition of the IL-4/STAT6 signaling pathway. These results indicate that coal tar enhances skin barrier function and dampens keratinocyte response to the major cytokines involved in AD, and point to a key role of the AHR signaling pathway in the molecular mechanism by which the oldest known drug in dermatology corrects epidermal abnormalities in a common skin disease.

Results

AHR regulates coal tar–induced epidermal gene and protein expression. First, we investigated the potential of coal tar to activate AHR using cultured adult primary human keratinocytes. A coal tar formulation was prepared in culture medium and various concentrations were tested for toxicity. After 48 hours of treatment, none of the concentrations tested resulted in enhanced cell death (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI65642DS1). In a variety of cells, it has been shown that upon ligand binding, AHR translocates to the nucleus. We indeed observed that coal tar caused AHR to relocate from the cytoplasm to the nucleus within 4 hours (Figure 1A), and we found a significant increase over time in the AHR nucleus/cytoplasm ratio upon TCDD and coal tar stimulation (Figure 1B). Preincubation of the antibody with recombinant AHR before immunofluorescence analysis completely eliminated nuclear staining of the coal tar–treated cells, which indicates the specificity of the immunofluorescence staining (Supplemental Figure 1B). We found a strong time- and concentration-dependent increase in expression of typical AHR-responsive genes such as CYP1A1 and CYP1B1 in coal tar–treated keratinocytes, while expression of CYP2E1 (not an AHR target) was unaffected (Figure 1C and Supplemental Figure 2). The response of keratinocytes to coal tar was similar in magnitude to their response to TCDD, a dioxin-like compound used as a positive control. Aside from CYP450 target gene expression, we also observed a highly significant and concentration-dependent induction of the epidermal differentiation genes FLG, hornerin (HRNR), and involucrin (IVL) (Figure 1D), all pivotal for barrier function and all functionally related to AD (1, 2, 4, 7). For these genes, the induction rate achieved by high and intermediate coal tar concentrations was even greater than that achieved by TCDD. Other epidermal differentiation genes such as cystatin M/E (CST6), repentin (RPTN), and small proline-rich proteins 2A and 2B (SPRR2A and SPRR2B) were also induced after coal tar treatment, but to a lesser extent (Supplemental Figure 3). The induced mRNA expression was confirmed at the protein level by staining of coal tar–treated and untreated keratinocytes cultured on coverslips (Figure 1E) and by Western blotting (Figure 1F). Filaggrin protein expression in untreated keratinocytes was confined to a few multilayered foci, while in coal tar–treated keratinocytes, its expression was already present in large patches of the keratinocyte monolayer (Figure 1G). These results indicate that coal tar treatment induced filaggrin expression at early stages of keratinocyte differentiation.

To confirm the regulatory role of the AHR signaling pathway in the coal tar–mediated induction of epidermal differentiation genes, we studied the coal tar response after siRNA-mediated knockdown of AHR in keratinocytes. As shown in Figure 1H, a 79% decrease in AHR expression was achieved, with a concomitant 82% decrease in expression of its classical target gene CYP1A1. AHR knockdown also caused a similar decrease in coal tar–induced FLG and HRNR gene expression (Figure 1I) and other terminal differentiation genes (Supplemental Table 1). The induction rate after coal tar treatment and subsequent inhibition by AHR knockdown was most pronounced in terminal differentiation genes with a reported xenobiotic response element in their promoter region (17), such as FLG, HRNR, and filaggrin family member 2 (FLG2). In contrast, the early differentiation marker keratin 10, or the basal keratinocyte markers keratin 5 and keratin 14, were not affected by coal tar stimulation or AHR knockdown. Altogether, these data show that coal tar induces epidermal differentiation, which is regulated by the AHR signaling pathway.

Accelerated epidermal differentiation by coal tar treatment. Next, we used an organotypic human skin model (hereinafter referred to as “skin equivalents”) to evaluate the effects of coal tar during epidermal morphogenesis. We have previously shown that skin equivalents can be used successfully to mimic many aspects of normal epidermis or diseased epidermis (e.g., psoriasis and AD) (18, 19). After 3 days of submerged culture, the skin equivalents were lifted at the air-liquid interface and the culture medium was supplemented with coal tar. Already after 4 days of culture at the air-liquid interface, a fully developed epidermis was observed in coal tar–treated skin equivalents. The untreated skin equivalents required 10 days of air exposure to generate a well-developed epidermis (Figure 2A). From day 4 onward, the epidermis of coal tar–treated skin equivalents continuously produced terminally differentiated cells, resulting in a very thick layer of stratum corneum at the air-liquid interface on day 10 of culture (Figure 2A; bracket). To further examine the differentiation process of coal tar–treated skin equivalents, we analyzed protein expression at several time points using immunohistochemistry. Terminal differentiation markers, which are normally expressed from day 6 of air-exposed culture onward (20), were already expressed at high levels at day 4 in coal tar–treated skin equivalents. In particular, filaggrin, loricrin, and hornerin were barely present in the standard (control) cultures at day 4, while they were abundantly expressed in coal tar–treated skin equivalents (Figure 2, B and C). Therefore, we conclude that coal tar treatment in vitro results in accelerated epidermal differentiation, and is accompanied by induced expression of terminal differentiation proteins.

Coal tar increases filaggrin expression in skin equivalents and in lesional AD skin. Based on the observed association of AD with FLG haploinsufficiency and the increased risk of lower copy numbers of intragenic FLG repeats, it has been suggested that treatments resulting in increased filaggrin expression may have therapeutic value (3). To address this issue experimentally, we generated skin equivalents with keratinocytes from both healthy volunteers and AD patients, with a wild-type genotype or a heterozygous mutation (17). Such epidermal differentiation, and is accompanied by induced expression of terminal differentiation proteins.
in AD might be important in the pathophysiology of the disease (7), we also evaluated hornerin protein expression and found coal tar–mediated upregulated hornerin expression to be similar to that of filaggrin (Figure 3B). To correlate our in vitro model to the in vivo situation, we obtained skin biopsies from AD patients before and during coal tar therapy. In lesional AD skin, expression of filaggrin and hornerin was markedly lower and discontinuous, or even absent, compared with normal skin, as described previously (7). Already after 3 days of coal tar treatment, expression of these proteins was strongly induced in lesional AD skin, and after 7 days of coal tar therapy, a continuous expression pattern was observed (Figure 3C), resembling that of normal skin. We also analyzed other epidermal differentiation proteins and found strongly upregulated expression of loricrin and involucrin proteins both
in vitro and in vivo (Figure 3D), suggesting that AD patients may benefit from enhanced epidermal differentiation in general, as well as improved barrier function.

**Coal tar normalizes histopathological and molecular hallmarks of AD in skin equivalents.** Th2 cytokines like IL-4 and IL-13 induce spongiosis and apoptosis in skin equivalents, probably via STAT6 signaling (18). These cytokines were also shown to downregulate involucrin and loricin expression via STAT6 (4). Furthermore, the constitutive expression of STAT6 in transgenic mice has been demonstrated to lead to an increased risk of developing AD (5). We therefore stimulated skin equivalents with IL-4 and IL-13 to induce an AD phenotype in vitro as previously described (18), and tested the effect of coal tar. We found that coal tar treatment prevented the occurrence of spongiosis and apoptosis and could “cure” these symptoms in a therapeutic experimental design; both simultaneous addition of Th2 cytokines and coal tar, and coal tar treatment after Th2 stimulation, resulted in improvement of the phenotype (Figure 4, A and B). Although Th2 cytokine–mediated apoptosis was reported to be associated with increased expression of FAS (21), we did not observe downregulated FAS levels after coal tar treatment (Figure 4C). In our skin equivalent model, we confirmed the reported Th2 cytokine–mediated downregulation of epidermal differentiation proteins (4) and found coal tar treatment to restore both mRNA (Figure 4D) and protein expression (Figure 4, E and F). This was also confirmed in coal tar–treated AD patients in vivo (Figure 3D). Eosinophilia has been associated with AD, and the degree of eosinophilia correlates with AD severity (22). CCL26, or eotaxin-3, a chemokine for eosinophils, is upregulated in keratinocytes by IL-4 via the STAT6 signaling pathway (23). Our results have shown coal tar to diminish the downstream effects of IL-4, therefore we analyzed CCL26 levels in our AD skin equivalent model after coal tar treatment. The highly upregulated CCL26 expression levels in skin equivalents after Th2 cytokine stimulation were completely normalized after coal tar treatment (Figure 4G), indicating that coal tar directly targets the innate immune system by interfering with the keratinocyte response following Th2 cytokine stimulation.

**Coal tar interferes with the Th2 cytokine–mediated STAT6 signaling cascade.** Since most of the aforementioned effects of Th2 cytokine stimulation in keratinocytes act via STAT6 signaling, we hypothesized that coal tar interferes with this signaling cascade. Both phosphorylated STAT6 (pSTAT6) and total STAT6 levels were determined using Western blot analysis, which revealed that stimulation with IL-4 and IL-13 induced STAT6 phosphorylation (Figure 5A). Thereafter, we supplemented Th2 cytokine–stimulated keratinocytes with coal tar for 6 hours and observed markedly reduced pSTAT6 levels in these cell lysates (Figure 5A). pSTAT6 is a known substrate for protein tyrosine phosphatase 1B (PTPN1) (24). In addition, oxidative stress induced by Th2 cytokine stimulation in keratinocytes results in oxidative inactivation of PTPN1, and thus sustained STAT6 phosphorylation (25). We therefore hypothesized that the molecular mechanism of coal tar–mediated inhibition of STAT6 phosphorylation could, at least in part, act via inhibition of the oxidative inactivation of PTPN1. AHR is known as a sensor of the redox system against oxidative stress and regulates nuclear factor erythroid 2–related factor 2 (NRF2), a master switch of the redox machinery (26). We found coal tar to induce both nuclear translocation of NRF2 (Figure 5B) and subsequent induction of its target gene,
NAD(P)H quinone oxidoreductase 1 (NQO1) in keratinocytes (Figure 5C). Remarkably, no induction of NQO1 was observed in keratinocytes following TCDD exposure. Upon AHR knockdown, induction of NQO1 expression was inhibited, indicating the regulatory role of AHR in NRF2-mediated signaling in keratinocytes (Figure 5D). Our data indicate that coal tar activates the antioxidative stress pathway via NRF2 signaling, which might inhibit Th2-induced oxidative stress and result in STAT6 dephosphorylation and abrogation of the inflammatory Th2-mediated STAT6 signaling cascade in keratinocytes (Figure 6).

Discussion

AD is a highly prevalent inflammatory skin disease caused by skin barrier dysfunction and chronic immune activation (27, 28).

Figure 3

Restored filaggrin and hornerin protein expression by coal tar. Skin equivalents generated from keratinocytes from AD patients harboring a heterozygous FLG mutation (FLG<sup>+/–</sup>; n = 3) and healthy control wild-type (WT; n = 3) keratinocytes were treated with coal tar during the last 3 days of air-liquid interface culture. Skin equivalents were stained for (A) filaggrin, and (B) hornerin protein expression analysis. (C) Immunohistochemical analysis of filaggrin and hornerin protein expression in skin biopsies from AD patients receiving coal tar therapy (n = 3). Scale bar: 100 μM. (D) Immunohistochemical analysis of loricrin and involucrin protein expression in skin biopsies from AD patients receiving coal tar therapy (n = 3). Scale bar: 100 μM.
No mechanism-based therapies are available for AD patients and treatment is mainly focused on symptom relief, predominantly targeting dry skin, itch, and inflammation with emollients and corticosteroids. Since the seminal work of the McLean Lab has underscored the importance of skin barrier abnormalities as an etiological factor in AD, we focused on the effects of coal tar on the epidermal biology of this disease (1, 3, 8). Our results reveal a key role of the AHR signaling pathway in breaching the vicious
circle of chronic inflammation by improving skin barrier function and interfering with Th2 cytokine signaling and downstream pathological processes.

The immunopathogenesis of AD has been studied directly on patients or in animal models based on allergic immune responses (29–31). We have recently developed in vitro organotypic models for psoriasis and AD (18, 19), and have shown that the application of genetic techniques in these models could be used to study epidermal aspects of diseases (32). Others showed that FLG knockdown in human skin equivalents could mimic AD skin by impaired epidermal differentiation and barrier dysfunction (33, 34). We have chosen keratinocyte-only human skin equivalents to evaluate the effect of coal tar on the epidermis, thereby excluding the possible effects of coal tar and subsequent AHR activation on other skin cell types such as fibroblasts and immune cells, as these cells could in turn affect epidermal processes. Genetic approaches to modify keratinocyte gene expression were introduced in this system by using an siRNA knockdown approach to prove the role of AHR, or by using patient-derived keratinocytes harboring one FLG-null allele. These findings illustrate the power of skin equivalents to study human skin diseases and, to a certain extent, to replace mouse models.

In the early seventies, attempts were made to unravel the mechanism of action of coal tar therapy. Suppression of DNA synthesis (35, 36) and induction of a granular layer using the mouse tail test (37, 38) have been reported. The mouse tail test has previously demonstrated the effects of coal tar on epidermal differentiation. Since the discovery of FLG mutations in AD, it is now widely accepted that epidermal barrier dysfunction is key in the pathophysiology of this disease. These new insights are strongly guiding current efforts in AD research and therapy development. The accelerated filaggrin expression and barrier function in fetal mouse skin due to dioxin-mediated AHR activation (17, 39) suggested to us that there is a potential role for AHR in AD treatment. Here, we show that coal tar activates AHR signaling and found that AHR regulates and induces epidermal differentiation and stimulates filaggrin expression in keratinocytes harboring a monoallelic FLG loss-of-function mutation. These findings may have a major impact on the reappraisal of coal tar therapy, which is gradually being abandoned by dermatologists due to its cosmetic side effects, safety concerns, and a hitherto unknown mechanism of action.

The AHR signaling pathway is currently of great interest. Though it was previously only considered to be related to immunotoxicity, recent findings have underscored this pathway’s physiological role. AHR appears to be predominantly involved in the development and function of the immune system (14, 40–43). Genetic approaches in mice, however, have also revealed that AHR signaling plays a role in epidermal pathophysiology. Deficiency or constitutive expression of AHR interaction partners ARNT (44, 45) and NRF2 (46), respectively, has detrimental effects on epidermal differentiation and barrier function, whereas AHR transgenic mice develop inflammatory skin lesions with hyperkeratinization (47). All these studies show that disturbance of normal AHR signaling, either by genetic approaches or TCDD exposure, leads to epidermal abnormalities. Our study is the first to demonstrate the beneficial therapeutic effects of AHR activation on epidermal differentiation and barrier function in a skin disease characterized by low levels of terminal differentiation proteins and the resultant poor barrier function. The

Figure 5

STAT6 dephosphorylation and NRF2 activation by coal tar. (A) Western blot analysis of phosphorylated STAT6 (pSTAT6) and total STAT6 levels in lysates of 2 keratinocyte donors. Keratinocytes were stimulated for 12 hours with differentiation medium (–/–), Th2 cytokines (+/–), or 2% coal tar (–/+), and with Th2 cytokines during the first 6 hours, followed by the addition of 2% coal tar and stimulation for another 6 hours (+/+). Thin black line indicates that the lanes were run on the same gel, but were noncontiguous. (B) Immunofluorescence staining of NRF2 in coal tar–treated or untreated keratinocytes. Nuclei were counterstained using DAPI. Images are representative of 3 keratinocyte donors. Original magnification, ×400. (C) qPCR analysis of NRF2 and NQO1 mRNA expression levels in keratinocytes. Expression levels are relative to untreated (control) keratinocytes. *P = 0.002 relative to control condition. Bars indicate mean ± SEM (n = 3). qPCR analysis of mRNA expression levels of AHR (D) and NQO1 (E) after siRNA-mediated AHR knockdown and subsequent coal tar stimulation by qPCR analysis. Expression levels are relative to mock-treated, coal tar–stimulated keratinocytes. **P = 0.006 and ***P = 0.032, relative to mock-treated keratinocytes. Bars indicate mean ± SEM (n = 3).
therapeutic use of AHR ligands appears controversial in light of the complete ban on all AHR ligands instituted by the pharmaceutical industry because of the widely known dioxin-related toxic effects of AHR activation. However, recent interest in the AHR signaling pathway has led to a variety of reports describing diverse downstream effects by different exogenous and endogenous AHR agonists. Nowadays, therefore, it is recognized that the specific ligand-receptor interaction determines the downstream effects of AHR activation, and that it is not merely a matter of drug potency. Hence, CYP1A1 induction and/or AHR activation are not synonymous with dioxin-like toxicity.

In addition to the recently uncovered role of NRF2 in the defense against oxidative stress, and it is therefore proposed as a potential pharmacological target for chemoprevention (53). NRF2 has been linked to the AHR signaling pathway mostly in mice liver tissue after dioxin exposure (54). Here we show that coal tar, but not TCDD, induces NRF2 activation and subsequent expression of NQO1 in human keratinocytes. Upon knockdown of AHR, significantly less NQO1 was expressed, indicating an interaction between AHR and NRF2 in human keratinocytes. The discrepancy between the previously reported TCDD-induced NQO1 levels and the lack of such induction in keratinocytes is in itself not unprecedented, and may be explained by species and cell type differences and ligand-specific effects (55). Therefore, the induction of a protective mechanism (NQO1 induction) in keratinocytes after coal tar exposure might explain the lack of toxicity and carcinogenicity associated with the medicinal use of coal tar (56), while AHR activation by TCDD leads to severe symptoms such as chloracne and immunotoxicity, as well as an increased risk of cancer.

Although coal tar therapy is the oldest known dermatological treatment, questions still remain regarding its safety and possible carcinogenicity (57). A comprehensive study by Roelofzen et al. in 2010 (56) did not find a relation between an increased risk of skin or non-skin malignancies and coal tar therapy in a large cohort of 13,200 psoriasis and eczema patients. AHR ligands are currently excluded from drug discovery and development pipelines due to the exposure-related toxicity and carcinogenicity of some of its known ligands, such as dioxin and benzo[a]pyrene. The emerging evidence of a more physiological role of AHR, and the therapeutic effect of AHR activation herein described, suggest that it might be the right time to reconsider whether it is justifiable to exclude AHR as a pharmacological target.

**Methods**

**Coal tar formulations.** Pix Licantrix (medicinal coal tar) obtained from BUFA (Spruyt Hillen) was added to keratinocyte differentiation medium supplemented with 0.5 mg/ml delipidized BSA at an initial concentration of 2% (v/v). After overnight vortexing at room temperature, the saturated coal tar suspension was centrifuged and the supernatant was sterilized by 0.2 μm filtration. This solution was diluted 10 times in culture medium to obtain the highest nontoxic concentration (this condition was further designated...
as the “2%” concentration in all experiments). Further dilutions in culture medium were made from this solution. Coal tar is known to be composed of a large number (>10,000) of organic compounds, many of which are nonpolar and practically insoluble in aqueous solution. The presence of delipidized BSA in the medium is likely to have facilitated the solubility of part of the nonpolar fraction, but no attempt was made to quantify the fraction of the crude coal tar that was actually dissolved.

Toxicity testing. Cellular toxicity was measured using cell supernatant and lactate dehydrogenase analysis according to the manufacturer’s recommendations (Roche). Percentage of cell death, which was set at 100%, was relative to 1% Triton-X-1%PBS-treated keratinocytes.

Submerged keratinocyte culture. Keratinocytes were isolated from human abdominal skin derived from donors who underwent surgery for abdominal wall correction, as previously described (17). For submerged culture, keratinocytes were cultured in keratinocyte growth medium (Lonza) and differentiated by depletion of growth factors, as described previously (58). Coal tar was supplemented during the differentiation phase at indicated concentrations. The study was conducted in accordance with Declaration of Helsinki principles.

Skin equivalent development. Human skin equivalents were generated using de-epidermized dermis, as described previously, and seeded with 10⁶ human primary keratinocytes (17). Skin equivalents were cultured submerged for 3 days, after which the medium level was decreased and equivalents were cultured at the air-liquid interface at indicated time points in the presence of coal tar.

Skin morphology. Six-micrometer sections were obtained from paraffin-embedded, formalin-fixed skin equivalents or skin biopsies and were stained with H&E.

Indirect immunostaining of skin equivalents or biopsies and keratinocyte cultures. Indirect immunoperoxidase technique with avidin-biotin complex enhancement (Vectastain; Vector Laboratories) was used to analyze epidermal differentiation. The following antibodies were used: antibodies against filaggrin (1:6000; Biomedical Technologies Inc.); loricrin (1:500; BabCO); involucrin (1:50; Mon150; generated by our lab, van Duijnhoven [1992], University of Geneva, Switzerland); and hornerin (antibody was raised against full-length recombinant HRNR repeat domains, 1:200; Sigma-Aldrich) was used to control equal protein loading. To control for filaggrin and loricrin detection. For submerged cultured keratinocyte lysates, actin antibody (Sigma-Aldrich) was used to control equal protein loading. To control equal loading of the insoluble fraction from skin equivalent epidermis, Coomassie Blue staining of gels and Poncova-S S staining of blots were used (data not shown). Prior to immunoblotting, proteins were separated by SDS page and transferred to PVDF membranes using the NuPAGE system (Life Technologies). LumiGLO (Cell Signaling Technology Inc.) was used for chemiluminescent detection by the Bio-Rad Universal Hood Gel Imager (Bio-Rad Laboratories).

Toxicity testing. Cellular toxicity was measured using cell supernatant and lactate dehydrogenase analysis according to the manufacturer’s protocol. For analysis of the p.R501X (c.1501C>T) and c.2282del4 mutations, we used the previously published primers FliH1F3/RPT1P6 and RPT1P7/RPT2P1, respectively (62). DNA (50 ng) was diluted in PCR reaction buffer containing 1.6 mM MgCl₂ and Platinum Taq Polymerase (Invitrogen; Life Technologies). PCR conditions were as follows: 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds; 58°C for 45 seconds; 72°C for 75 seconds; and the final extension step at 72°C for 7 minutes. The amplified PCR products were resolved on a 1.5% agarose gel, purified by using the NucleoSpin Gel and PCR Clean-up kit (BIOKÉ), and sequenced using dye termination chemistry on a 3730 DNA analyzer (Applied Biosystems; Life Technologies).

Western blot analysis. Keratinocytes or epidermal sheets from skin equivalents separated by disperse treatment were lysed in RIPA lysis buffer. After a single cycle of freeze-thawing, the lysates were centrifuged at maximum speed for 10 minutes at 4°C. The supernatant was used for immunoblotting. Total protein concentration of the supernatant was determined, and a total of 12.5 μg protein was loaded for each sample. The pellet containing the insoluble fraction was resuspended in sample buffer, sonicated, and boiled for 5 minutes and used for filaggrin and loricrin detection. For submerged cultured keratinocyte lysates, actin antibody (Sigma-Aldrich) was used to control equal protein loading. To control equal loading of the insoluble fraction from skin equivalent epidermis, Coomassie Blue staining of gels and Poncova-S S staining of blots were used (data not shown). Prior to immunoblotting, proteins were separated by SDS page and transferred to PVDF membranes using the NuPAGE system (Life Technologies). LumiGLO (Cell Signaling Technology Inc.) was used for chemiluminescent detection by the Bio-Rad Universal Hood Gel Imager (Bio-Rad Laboratories).

FLG mutation analysis. Genomic DNA was extracted from saliva samples using the Oragene kit (DNA Genotek Inc.) according to the manufacturer’s protocol. For analysis of the p.R501X (c.1501C>T) and c.2282del4 mutations, we used the previously published primers FliH1F3/RPT1P6 and RPT1P7/RPT2P1, respectively (62). DNA (50 ng) was diluted in PCR reaction buffer containing 1.6 mM MgCl₂ and Platinum Taq Polymerase (Invitrogen; Life Technologies). PCR conditions were as follows: 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds; 58°C for 45 seconds; 72°C for 75 seconds; and the final extension step at 72°C for 7 minutes. The amplified PCR products were resolved on a 1.5% agarose gel, purified by using the NucleoSpin Gel and PCR Clean-up kit (BIOKÉ), and sequenced using dye termination chemistry on a 3730 DNA analyzer (Applied Biosystems; Life Technologies).

Study approval. The study was approved by the local medical ethical committee and was conducted according to Declaration of Helsinki principles.

Informed consent was obtained from all patients and healthy volunteers.

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