

Histamine Exerts Multiple Effects on Expression of Genes Associated With Epidermal Barrier Function

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■ Abstract

Background: The role of epidermal barrier genes in the pathogenesis of atopic skin inflammation has recently been highlighted. Cytokines that are abundant in the skin during inflammation have been shown to exert various effects on the expression of barrier genes, although the role of histamine in this area of skin biology is not yet fully understood.

Objective: To assess the effect of stimulation with histamine on keratinocytes by analysis of the pathways involved in epidermal barrier integrity.

Material and Methods: We performed a gene expression analysis of histamine-stimulated keratinocytes. Functional changes were tested using the dye penetration assay. Differential changes in filaggrin and the filaggrin-processing enzyme bleomycin hydrolase (BLMH) were validated at the protein level, and expression was also assessed in filaggrin knock-down keratinocytes.

Results: Histamine altered expression of multiple barrier genes. Expression of filaggrin was downregulated, as was that of other markers, thus suggesting the presence of delayed/aberrant keratinocyte differentiation. Expression of genes involved in cellular adhesiveness and genes of protease expression was dysregulated, but expression of protease inhibitors was increased. BLMH was upregulated in keratinocytes subjected to histamine and filaggrin knockdown.

Conclusions: Histamine exerts a dual effect on epidermal barrier genes; it suppresses keratinocyte differentiation and dysregulates genes of cellular adhesiveness, although it induces genes contributing to stratum corneum function. Upregulation of BLMH and protease inhibitors could support maintenance of the permeability barrier by enhanced generation of moisturizing compounds and suppressed desquamation. In contrast, in the case of stratum corneum damage, histamine could enhance transcutaneous sensitization.

Key words: Epidermis. Epidermal differentiation. Skin barrier. Filaggrin. Wound healing. Histamine.

■ Resumen

Antecedentes: Recientemente se ha comprobado el papel de los genes de la barrera epidérmica en la patogénesis de la inflamación de la piel atópica. Se ha demostrado la presencia de abundantes citocinas en la piel durante la inflamación, que ejercen diferentes efectos sobre la expresión de los genes de la barrera, si bien el papel de la histamina en la biología de la piel no se conoce bien.

Objetivo: El objetivo de este estudio fue analizar el efecto de la estimulación de los queratinocitos por la histamina centrándonos en las múltiples vías involucradas en mantener la integridad de la barrera epidérmica.

Material y Métodos: Para ello se realizó un análisis de la expresión de los genes de queratinocitos estimulados con histamina y en queratinocitos sin filagrina. Los cambios funcionales fueron estudiados mediante un ensayo de penetración de colorante. Se analizaron los cambios diferenciales en la filagrina y en la enzima procesadora de la filagrina bleomicina hidrolasa (BLMH)

Resultados: En cuanto a los resultados obtenidos se observó que la histamina fue capaz de alterar la expresión de numerosos genes de la barrera cutánea. La regulación a la baja de la filagrina observada, así como el patrón de expresión de otros marcadores sugiere una retarda y/o aberrante diferenciación de los queratinocitos provocada por la histamina. La expresión de genes involucrados en la adherencia celular y en la expresión de proteasas se altera, con un aumento de la expresión de inhibidores de las proteasas. La hidrolasa de la bleomicina se incrementa en los queratinocitos tratados con histamina y en queratinocitos sin filagrina.

Conclusiones: La histamina ejerce un efecto dual sobre los genes de la barrera epidérmica, suprime la diferenciación de los queratinocitos y disregula la adhesividad celular, por otra parte induce la expresión de genes que contribuyen a la función del estrato córneo.

La regulación al alta de la hidrolasa de la bleomicina y los inhibidores de las proteasas podrían mantener la permeabilidad de la barrera cutánea facilitando la generación de componentes humectantes y suprimir la descamación. En contraste, sobre el estrato córneo la histamina provoca un daño que podría facilitar la sensibilización transcutánea.

Palabras clave: Epidermis. Diferenciación epidérmica. Barrera cutánea. Filagrina. Reparación de heridas. Histamina.

Introduction

Atopic eczema is a chronic inflammatory skin disease in which sensitization to multiple common environmental allergens is common. The role of the epidermal barrier dysfunction in induction of sensitization and development of skin inflammation has been well documented (reviewed in [1]) and suggests both genetic predisposition and acquired insufficiency of various components of the epidermal barrier. Multiple barrier genes have been shown to play a key role in pathogenesis; *FLG* mutations have been reported as the most reproducible genetic susceptibility factors in cohorts around the world [2]. However, apart from inherited traits, acquired predisposition as a result of the interaction between incoming immune cells and the epidermis [3-7] seems to have an important role in pathogenesis.

Mediators released in the skin during inflammation exert a pronounced impact on barrier permeability and other vital epidermal functions. We recently proposed that key cytokines in early inflammation (ie, those involved in the activation of innate and acquired immune defenses and wound healing) transiently disturb epidermal barrier function in order to facilitate access of both inflammatory cells and humoral factors to inflammatory sites and aid wound closure [8]. While these temporal changes are necessary to reconstitute homeostasis of the skin, the altered cytokine milieu could at the same time promote transcutaneous allergen exposure, potentially leading to sensitization to multiple environmental allergens.

Histamine is a major mediator at inflammatory sites, especially during allergic reactions [9-12]. Keratinocytes express the histamine receptors HRH1, HRH2, and HRH4 [13-15], which induce signals leading to proinflammatory outcome in those cells after exposure to the amine. Circumstantial evidence linking inflammation in the lungs and at mucosal sites to allergic sensitization during infection in human and animal studies [16-25] indicates the possibility of a similar correlation in the skin. Therefore, we hypothesized that exposure to histamine could lead to altered expression of multiple genes associated with epidermal permeability barrier function and, thus, increased susceptibility to transcutaneous sensitization during inflammation.

Materials and Methods

Cells

HaCaT cells were cultured in DMEM medium supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin. Keratinocytes (Lonza, TCS Cellworks) were maintained in KGM Gold medium (Lonza) with 0.06 mM calcium; the cells used for the experiments were of early passage. All cells were maintained at 37°C with 5% CO₂ and 95% relative humidity. To induce differentiation and filaggrin expression, the calcium concentration was increased to 1.5 mM for up to 5 days before the experiments. Histamine was added at 1 pg/mL to 1 µg/mL for 24/48 hours. Culture medium without histamine was used as a control throughout. Stained cells were grown on culture slides (Beckton Dickinson).

Microarray, Gene Ontology Analysis, and Quantitative PCR

Histamine was added to HaCaT cultures at a concentration of 1 µg/mL at 80-90% confluence; experiments were carried out in triplicate. RNA was extracted after 24 hours (RNeasy Plus, Qiagen); microarray acquisition was performed by Service XS (Holland) on a BeadArray platform (Illumina). The data were submitted to Gene Expression Omnibus (GSE41585). Control values obtained from setups comprising medium without histamine are given as baseline and histamine-treated values as a log₂-fold change over baseline (baseline=0). qPCR was carried out using the RNA-to-Ct 1-step kit with *FLG*/*GAPDH* primers (Hs00856927_g1 and Hs99999905_m1; Applied Biosystems).

Western Blot

Cells were lysed with RIPA buffer (Cell Signaling Technology) containing cOmplete Protease Inhibitor Cocktail (Roche). Lysates were fractionated on NuPage 7% Tris-Acetate or 12% Bis-Tris gels (life technologies). Western blot assays (PVDF, iBlot system, life technologies) were carried out using antifilaggrin (AKH-1 or H-300, Santa Cruz) and anti-GAPDH antibodies (AbDSerotech).

Stratified Epidermal Models and Dye Penetration Assay

Underdeveloped epidermal models (EPI-201, EPI-200-1s, EPI-200-3s; MatTek) at various stages of differentiation grown at the air-liquid interface in manufacturer-supplied medium as per protocol were cultured in histamine-enriched (1 µg/mL) or control medium for 5 days. For the dye penetration assay, 1 mM luciferase yellow was applied topically and inserts were incubated for up to 4 hours. Inserts were frozen in OCT and sectioned at a thickness of 4 µm.

Immunocytochemistry/Immunohistochemistry

The antibodies used for staining cells in culture slides and sectioned stratified epidermal models were anti-HRH1, anti-HRH2, anti-HRH3, anti-HRH4, anti-*FLG* (FLG01, Abcam), anti-BLMH (Abcam), isotype control mouse (Becton Dickinson), and rabbit (Invitrogen). Staining was performed using the EnVision+ system (Dako).

FLG Knockdown

HaCaT keratinocytes were transduced with either anti-*FLG* or control shRNA using a lentiviral system (Santa Cruz). Polybrene (Santa Cruz) was added to increase binding, and the cells were selected with puromycin-containing medium. Knockdown of RNA and protein was confirmed (data not shown).

Data Analysis

Microarray data were normalized with lumi [26] and analyzed with LIMMA [27]. Differential expression was identified at a cutoff log₂FC ±0.5 (adjusted *P*<.05), which we have used successfully in previous studies [13] where

we further validated the changes at the protein level. The remaining data were analyzed with Prism 6 software. The Mann-Whitney test was used.

Results

Expression of Histamine Receptors by Primary and Immortalized Keratinocytes

Using immunocytochemistry, we investigated the expression of the histamine receptors HRH1-HRH4 in both HaCaT cells and normal human epidermal keratinocytes. The results indicated that both cell types expressed the same receptors, namely, HRH1, HRH2, and HRH4. HRH3 was not expressed (Figure 1). This finding is in agreement with previously published data [13-15]. We also observed that expression of HRH4 was strongest, followed by expression of HRH2 and HRH1; the expression of these receptors did not seem to be calcium-dependent (Figure 1, low-high calcium data).

Histamine Dysregulates the Protease/Protease Inhibitor Axis

Maintenance of the balance between stratum corneum proteases and their inhibitors is an important mechanism that is responsible for shedding of corneocytes at the outer layers of the epidermis; any alteration in this mechanism can have potentially detrimental consequences. mRNA expression of urokinase and kallikrein 7 was upregulated, whereas expression of kallikrein 11 was downregulated (Figure 2A). In contrast, all significantly changed protease inhibitors were highly upregulated. Specifically, we observed a uniform increase in the expression of SERPIN inhibitors and secretory leukocyte

protease inhibitor (Figure 2B). However, we observed no differences in the expression of genes related to ceramide synthesis (data not shown).

Histamine Alters the Expression of Genes Involved in the Formation of Cellular Junctions

Enhanced migratory behavior of both innate and adaptive immune cells during inflammation requires decreased adhesion [28,29] by specific junctional structures. Therefore, we hypothesized that histamine alters the expression of related keratinocyte genes with a direct link to changes in the permeability barrier of live epidermis. Indeed, in our microarray dataset, we identified alteration of expression of many genes involved in adhesion (Table 1). In addition, several important genes, such as ZO-1, were downregulated at the level just below the accepted cutoff of $\log_2FC \pm 0.5$, although the differences remained significant in terms of the adjusted *P* value (data not shown). Taken together, our results show that histamine dysregulates expression of genes involved in the formation of junctions between keratinocytes.

Histamine Affects mRNA Expression of Keratins and Proteins of the Cornified Envelope

Keratinocyte differentiation and programmed death are essential for the formation of a mechanically robust cornified envelope; the expression of genes involved in differentiation has been shown to be critically important in supporting the barrier function of the epidermis. Differentiation-related proteins have been implicated in both genetically determined and acquired barrier defects [8,30,31]; therefore, we investigated whether histamine affects their expression in our model. First, we compared the mRNA level of the keratinocyte proteins that

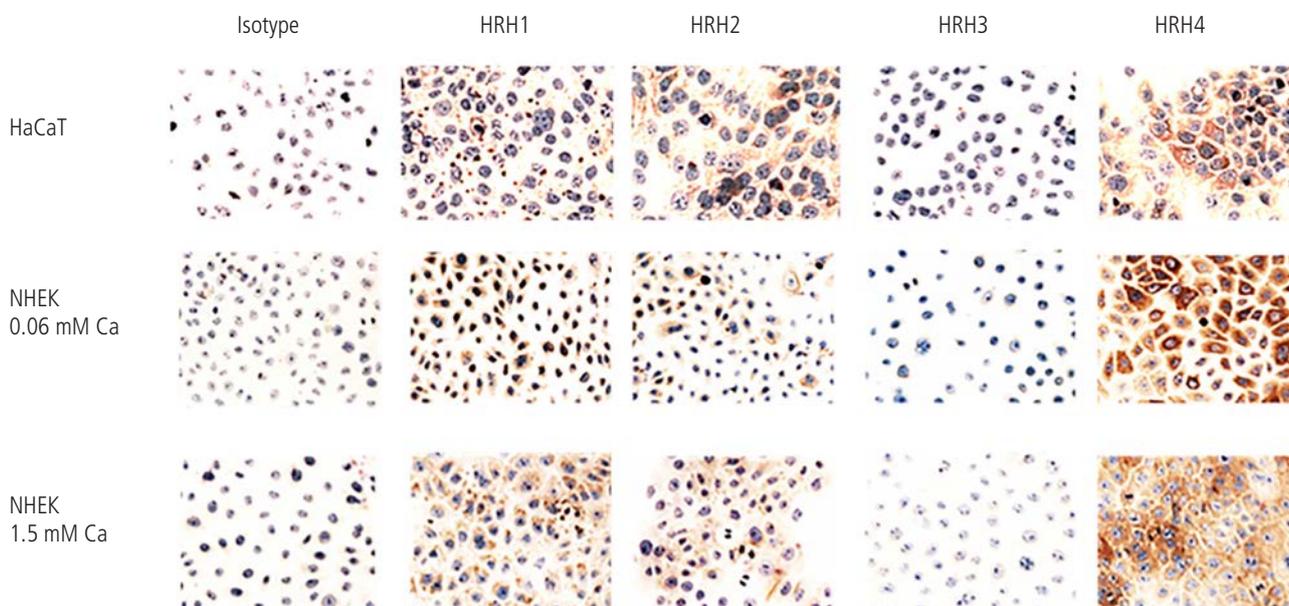


Figure 1. Expression of histamine receptors HRH1-HRH4 by primary and immortalized keratinocytes.

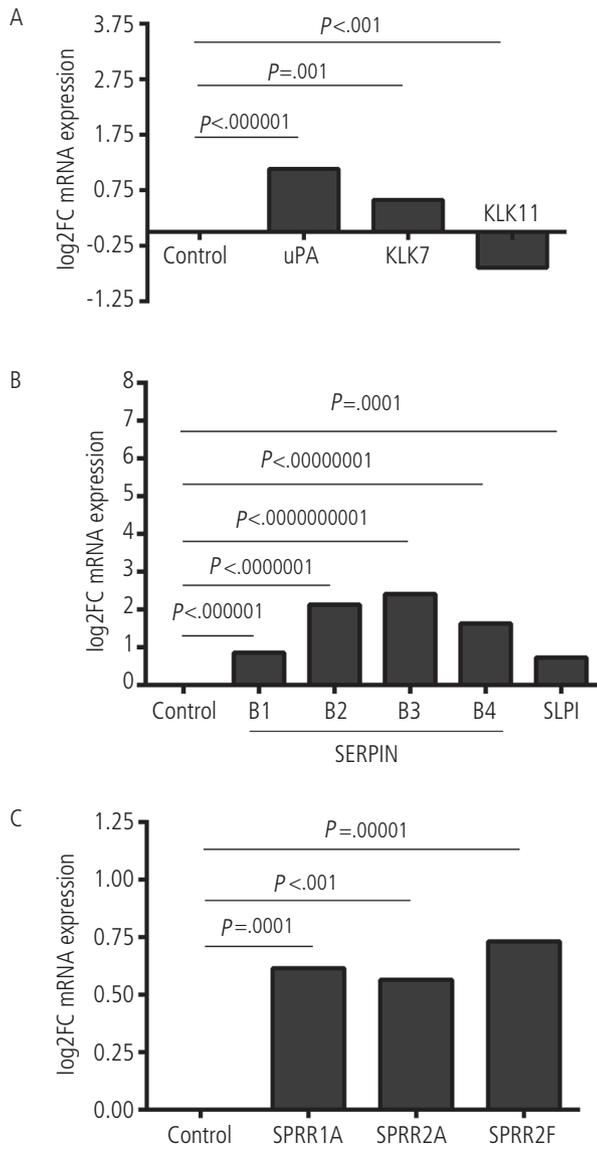


Figure 2. Histamine-mediated changes of expression of genes of the protease/protease inhibitor axis and SPRR family members. HaCaT cells were exposed to histamine, and RNA was extracted after 24 hours of incubation. Microarray data were acquired on the Illumina platform; A, Proteases. B, Protease inhibitors. C, SPRR proteins. Setups were carried out in triplicate; adjusted *P* values are shown; uPA indicates urokinase/plasminogen activator; KLK, kallikrein; B1-4 refer to SERPINS.

are major components of the cornified envelope (involucrin, loricrin, transglutaminases, small proline-rich region proteins [SPRR]) and observed increased expression of SPRR family members in histamine-exposed cultures (Figure 2C). We did not detect changes in loricrin/involucrin expression at the mRNA level after 24 hours (data not shown). In addition, many early keratins (K14, which is usually observed in mitotically active keratinocytes; K6, which is implicated in wound healing; activated/hyperproliferation-associated K16; and K17, which is expressed in skin appendages [Table 2]) were observed at increased mRNA expression levels, thus suggesting delayed/abnormal differentiation.

Finally, because of the extensive involvement of filaggrin in epidermal barrier formation [29], we investigated the effect of histamine on its expression. We identified 2 probes on the Illumina HT12 bead chip that were annotated as filaggrin-specific (ILMN_2134130 and ILMN_1720695). These differ in complementarity to the *FLG* sequence (lower vs higher, as per BLAST search; data not shown) and specificity (*FLG/HRNR* vs *FLG*, respectively). Since we obtained differential results with these probes, we subsequently validated our results by qPCR. This approach revealed no alteration of expression at the mRNA level (data not shown) at 24 hours, thus mirroring the results obtained using the Illumina probe, with a higher degree of specificity and complementarity to the *FLG* sequence.

Histamine Decreases Expression of Filaggrin Protein in Cultured Keratinocytes and Stratified Models

Availability of functional filaggrin monomers is only partly regulated by transcription of *FLG* and depends on additional factors involved in processing and degradation pathways. Therefore, we investigated this availability in more detail, despite the absence of differences at the mRNA level. We performed Western blot assays on primary keratinocytes, which underwent a calcium switch, and observed reduced expression of 37-kDa filaggrin monomer in keratinocytes exposed to histamine (Figure 3A and B). We also performed experiments in HaCaT keratinocytes using the more sensitive N-terminus-specific filaggrin antibody. We observed a decrease in the expression of both the 50-kDa band (likely N-terminal domain-containing) and higher-molecular-weight products. Based on those results, we showed that the additive effect could be observed for

Table 1. Histamine Alters Expression of Genes by Keratinocytes and Gene Transcript Variants Related to Cellular Adhesiveness

Gene	Symbol	Log ₂ FC	Adjusted <i>P</i> Value
Homo sapiens desmoglein 3 (pemphigus vulgaris antigen)	<i>DSG3</i>	-0.825	.00037
Homo sapiens claudin 8	<i>CLDN8</i>	-0.757	.00024
Homo sapiens desmoglein 2	<i>DSG2</i>	-0.585	.00025
Homo sapiens desmocollin 2, transcript variant Dsc2a	<i>DSC2</i>	-0.534	.00186
Homo sapiens gap junction protein, alpha 1	<i>GJA1</i>	0.949	.00007
Homo sapiens claudin 14, transcript variant 2	<i>CLDN14</i>	0.549	.00037
Homo sapiens integrin, alpha 6, transcript variant 2	<i>ITGA6</i>	0.533	.01111

Table 2. Changes of Expression of Keratin-Related Genes by Histamine as Reported by Microarray Analysis

Gene	Symbol	Log2FC	Adjusted <i>P</i> Value
Homo sapiens keratin 16	<i>KRT16</i>	2.099	.0000000001
PREDICTED: Homo sapiens similar to Keratin, type II cytoskeletal 8	<i>LOC643431</i>	1.882	.0000081792
Homo sapiens keratin 81	<i>KRT81</i>	1.861	.0000000001
Homo sapiens keratin 17	<i>KRT17</i>	1.369	.0000000024
PREDICTED: Homo sapiens similar to Keratin, type I cytoskeletal 18	<i>LOC340598</i>	0.811	.0000043351
PREDICTED: Homo sapiens keratin 18 pseudogene 13	<i>KRT18P13</i>	0.717	.0000701222
Homo sapiens keratin 6B	<i>KRT6B</i>	0.712	.0001263030
PREDICTED: Homo sapiens similar to keratin 8	<i>LOC100134794</i>	0.605	.0021913203
Homo sapiens keratin 14	<i>KRT14</i>	0.567	.0002375890

longer and at lower histamine concentrations (Figure 3C and data not shown). Finally, we also used epidermal models to verify the effect in stratified epidermis. These models were exposed to histamine during the final days of differentiation

at the air-liquid interface. Immunohistochemistry revealed a pronounced reduction in the reactivity of antifilaggrin antibodies within both granular layers and the stratum corneum (Figure 3D).

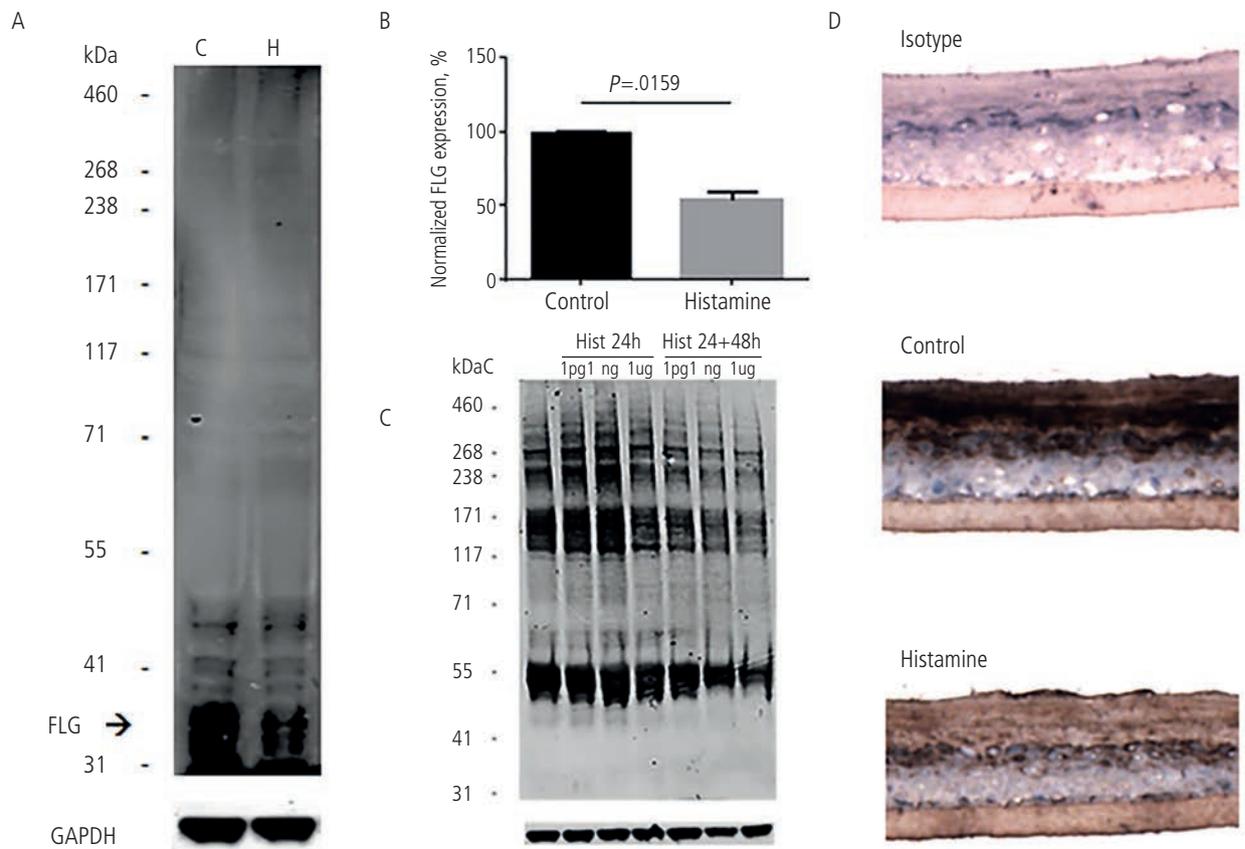


Figure 3. Histamine reduces expression of filaggrin in keratinocytes and stratified epidermal models. **A**, Primary keratinocytes were exposed to histamine following differentiation in high calcium-containing medium. Western blot was carried out with filaggrin monomer-specific antibody. **B**, Quantification of relative filaggrin expression vs GAPDH; cumulative data from 5 independent experiments. **C**, Histamine reduces expression of profilaggrin and processing products; histamine was added to HaCaT keratinocytes at a range of concentrations for either 1 or 2 days; Western blot was carried out with N-terminal domain-specific antibody. **D**, Histamine was added to the medium for the last 5 days of differentiation in the epidermal models, and filaggrin staining was performed; 3 organotypic models per group are shown.

Histamine Increases Expression of Bleomycin Hydroxylase

Since we observed that filaggrin—but not mRNA expression—was significantly downregulated at the protein level, we returned to our original microarray dataset to identify any changes in the expression of profilaggrin/filaggrin-processing and -degradation enzymes that could contribute to the observed effect. We observed upregulation of bleomycin hydrolase (BLMH, Figure 4A), an enzyme that is thought to be important in filaggrin monomer processing. To validate these findings, we further investigated expression of this enzyme by keratinocytes at the protein level. We observed that cultured cells were not uniformly positive, but that expression was marked in cellular aggregates; we also observed a large increase in their size (Figure 4B) and number after stimulation with histamine in both HaCaT cultures and primary keratinocyte cultures (Figure 4C and D).

Bleomycin Hydroxylase Expression Is Increased in Filaggrin-Insufficient Keratinocytes

Since filaggrin insufficiency seems to lead to additional functional consequences in keratinocytes [32-35], we were interested to see whether expression of BLMH is altered under such a circumstance. Therefore, we also evaluated BLMH expression in filaggrin knockdown keratinocytes (Figure 5A and data not shown), in which we observed significantly more BLMH^{high} keratinocyte aggregates than in control cultures (Figure 5B).

Barrier Function Is Maintained by the Stratum Corneum Under Exposure to Histamine

We observed altered expression of many components of the epidermal barrier, some of which seemed to be detrimental (others were considered potentially protective); therefore, we hypothesized that histamine could induce potentially

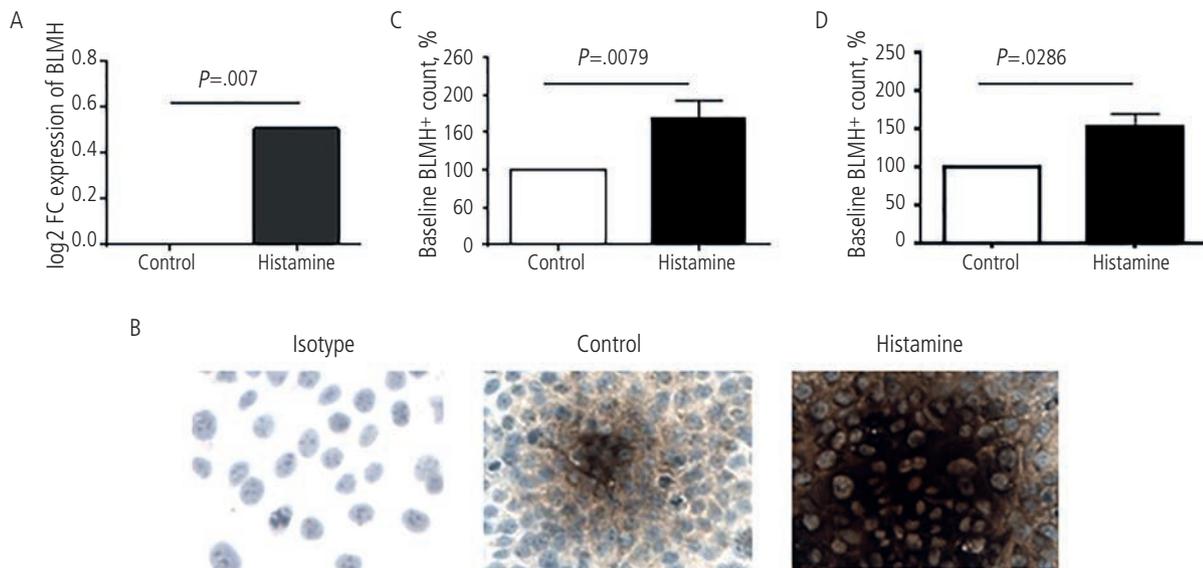


Figure 4. Histamine upregulates expression of BLMH in keratinocytes. A, Changes in BLMH mRNA expression under histamine stimulation; the adjusted *P* value is shown. B, immunocytochemistry staining of HaCaT cells showing differences in maximum sizes of BLMH^{high} cellular aggregates. C, Number of aggregates scored in HaCaT cells. D, Primary keratinocyte cultures; cumulative data are shown for at least 3 setups. BLMH indicates bleomycin hydrolase.

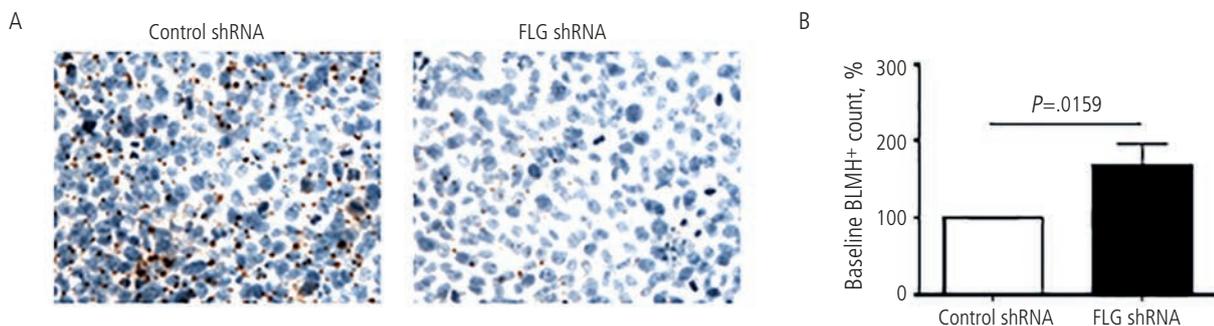


Figure 5. BLMH expression is upregulated in filaggrin-insufficient keratinocytes. A, Immunohistochemistry showing profilaggrin/filaggrin downregulation in stable filaggrin knockdown keratinocytes obtained using the shRNA lentiviral transduction system. B, Number of BLMH^{high} cellular aggregates was scored in filaggrin knockdown keratinocyte cultures; cumulative data of 3 setups are shown.

compensatory mechanisms in order to protect the permeability of the barrier in the case of inflammation. Therefore, we investigated a cumulative effect of those changes on the prevention of permeability. Luciferase yellow penetration assays on stratified, in vitro-grown epidermal tissues were chosen for this purpose; we repeated the assays in multiple models, which were characterized by periods of air-liquid interface differentiation (data not shown). However, regardless of the thickness of the stratum corneum, we did not observe any significant dye penetration, suggesting that this layer is able to maintain its protective role from very early stages of its formation. Similarly, we did not observe any dye penetration through to the live epidermal layers. The results were comparable across all the models tested, regardless of the period of air-liquid interface differentiation and stratum corneum thickness (data not shown), thus suggesting that the detrimental effect of exposure to histamine on the epidermal barrier would be limited to the layers below the stratum corneum; consequently, an impact would be observed in the case of injury such as scratching, which is typical in atopic skin inflammation.

Discussion

Acquired insufficiency of the epidermal barrier plays a role in the pathogenic mechanism of atopic skin disease, regardless of the presence of mutations in *FLG* or other skin barrier genes. We previously proposed that cytokines commonly involved in skin inflammation increase antigen penetration as an undesirable side effect of the temporal changes required to exert their critical roles during invasion by a pathogen or after trauma, thus acting as a double-edged sword [8]. In this study, we hypothesized that exposure to histamine could exert a similar function; indeed, we report an extensive effect on several of the constituents of a functional skin barrier.

The changes at the level of live epidermis that we observed after stimulation with histamine included altered cellular adhesiveness and reduced keratinocyte differentiation. Genes that support permeability barrier function mediated by the stratum corneum (ie, natural moisturizing factor [NMF]-generating enzyme, BLMH, and all differentially expressed protease inhibitors) were upregulated by this stimulation. The dye penetration assay confirmed that barrier permeability function is still protected in the presence of an intact stratum corneum.

Our results on keratinocyte differentiation and expression of tight junction genes are largely complementary to recently published data by Gschwandtner et al [36]. However, these authors did not investigate alterations in gene expression related to the function of the stratum corneum, namely, filaggrin processing and NMF-generation pathways or the protease/protease inhibitor axis. Here, we describe a significant effect of histamine on the expression of BLMH, a filaggrin-processing enzyme that has been implicated in the generation of NMF. While the reduced availability of filaggrin monomers could contribute to increased fragility of cornified envelopes, we believe that the effect of upregulation of BLMH on increased generation of hydrophilic amino acids may be of greater importance and compensate for this reduction, a notion is supported by previously documented findings. Specifically, the

severe/lethal phenotype of BLMH knockout mice highlights the indispensable role of BLMH-supported moisturization of the stratum corneum [37,38]. Moreover, the comparison between those animals and *FLG*-carrying flaky tail mice suggests that the major part of filaggrin function may in fact be NMF-mediated. However, there is no consensus on the expression of BLMH in atopic inflammation, since enzyme expression was increased in hyperkeratotic skin disorders [39], although downregulation in atopic skin has also been reported [40]. We believe that histamine-enhanced expression of this enzyme may be a major compensatory mechanism that reduces the detrimental effect of delayed keratinocyte differentiation and reduced cellular adhesion during inflammation processes in the skin. However, since we did not study the kinetics of profilaggrin mRNA beyond 24 hours after exposure to histamine, it is also possible that reduced synthesis could also contribute to the effect under prolonged stimulation, in agreement with the findings of Gschwandtner et al [36].

In addition, previously unreported changes in protease inhibitor expression could also contribute to the maintenance of stratum corneum-mediated barrier function. However, it is also possible that altered pH, combined with increased protease inhibitor expression, could lead to corneocyte retention, followed by detrimental scaling, as observed in X-linked ichthyosis [41].

Involvement of histamine receptors in the regulation of the effects observed is presently unclear. While H1 receptors were recently proposed to be important [36], both our unpublished data and previous findings establishing a role for both HRH1 and HRH2 antagonists in barrier recovery in mice [13] suggest that the mechanism may be more complex. This has implications for therapy.

Antihistamines may offer symptomatic relief, particularly in children, but they are not so effective in adults with atopic dermatitis. Nevertheless, taking the findings together, we consider that histamine is likely to exert a dual effect on the integrity of the epidermal barrier by increasing the permeability of live epidermal layers while supporting the function of the stratum corneum. The effect is likely to be important after penetrating trauma. Since intense pruritus and scratching are classically observed in atopic dermatitis, we believe that this effect could potentially lead to histamine-enhanced percutaneous sensitization in patients. Such a possibility would support the use of drugs that act within the histamine system, ideally by means of broad antihistamine blockade beyond H1 receptors and preferably in a topical formulation.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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