

Dexamethasone Promotes Keratinocyte Proliferation by Triggering Keratinocyte Growth Factor in Mast Cells

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Keywords

Glucocorticoids · Mast cells · Keratinocyte growth factor · Keratinocytes · Skin

Abstract

Background: The skin is a dynamic body organ that can be activated by both central and local hypothalamic-pituitary-adrenal axis systems. This phenomenon might be the crucial explanation why stress can cause relapse of chronic inflammatory skin diseases, such as psoriasis. Here, we determined the effects of mast cells on keratinocyte proliferation under stress hormone stimulation. **Methods:** We subcutaneously injected dexamethasone on the shaved back of mice and evaluated histological changes and keratinocyte growth factor (KGF) expression on dermal mast cells. Further, human mast cell line (HMC-1) and keratinocyte cell line (HaCaT) cells were treated with dexamethasone in vitro to observe the extent of proliferation and the expression of KGF. Finally, the supernatants of HMC-1 cells treated with dexamethasone were used for the culture of HaCaT cells to investigate the effect on proliferation. **Results:** We observed epidermal thickening in dexamethasone-injected mice, accompanied by an increase in the number of KGF-expressing dermal mast

cells. Similar to mouse dermal mast cells, KGF was highly expressed in the human mast cell line HMC-1 following stimulation with dexamethasone. Further, dexamethasone-treated mast cells promoted keratinocyte proliferation in vitro. However, the effects of mast cells on keratinocytes were significantly diminished in the presence of anti-KGF-blocking antibodies. **Conclusion:** Taken together, our results show that a stressful environment may disturb skin barrier homeostasis through mast cell-derived KGF expression.

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Introduction

Increasing evidence suggests stressful events are associated with the exacerbation of chronic inflammatory skin diseases, such as psoriasis [1, 2]. For example, more than half of the patients with psoriasis retrospectively report having experienced stressful life events before disease exacerbation [3, 4]. The stress response involves

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activation of both the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system, both of which interact with the immune system. Thus, stressful events could contribute to maintenance and exacerbation of chronic inflammatory diseases like psoriasis [5, 6]. Activation of the HPA axis results in increases in key stress hormones, including corticotropin-releasing hormone (CRH) in the hypothalamus, adrenocorticotrophic hormone (ACTH) in the anterior pituitary, and glucocorticoids (GC) in the adrenal cortex.

Interestingly, the skin has a fully functional peripheral equivalent of the HPA axis [6–8]. This means that the skin and hair follicles have their own local neuroendocrine system, which is capable of expressing CRH, ACTH, cortisol, and their reciprocal receptors. This is an efficient system because it allows the skin to respond to many different environmental stress factors, such as sunlight, injury, and chemicals when exposed. In fact, CRH expression and ACTH immunoreactivity are elevated in the epidermis, hair follicles, and sweat glands in some psoriatic patients [9]. ACTH can stimulate cortisol or corticosterone production in the skin, specifically in epidermal keratinocytes [10] and dermal fibroblasts [11]. One of the key regulatory mechanisms of inflammation and epidermal growth is cortisol, which can be synthesized in the human skin.

Mast cells in the skin are predominantly localized in the upper dermis and close to blood vessels, skin appendages, and neurons. Mast cells have been thought to undergo extensive activation, followed by mediator release, causing the symptoms of immediate-type allergy, such as urticaria. However, mast cells also undergo a slow, piecemeal degranulation or selective degranulation-independent mediator secretion [12, 13]. In addition to the well-known IgE-mediated activation of mast cells, these cells can be activated by several other endogenous factors, such as complement anaphylatoxins C3a and C5a [14] and stem cell factor [15].

In particular, CRH is also a direct activator of mast cells via the functional receptors CRH-R1 and CRH-R2 [16, 17]. Interestingly, cultured mast cells activated by CRH show selective secretion of vascular endothelial growth factor, a mediator that can play an essential role in angiogenesis in chronic inflammation [17]. Thus, endogenous hormonal factors could be causative agents that enable mast cells to modulate the microenvironment in the skin. Considering that stressful events have a critical impact on relapse in chronic inflammatory skin diseases, such as psoriasis, understanding the interaction between stress hormones and mast cells may provide new knowledge about skin immunity.

Maintaining a healthy skin barrier is the first requirement for the immune homeostasis of the skin. We hypothesized that continuous exposure to stress hormones, such as GC, may affect mast cells and subsequently modulate skin barrier. Thus, we investigated whether mast cells result in keratinocyte proliferation and mouse epidermal growth under GC stimulation and the relevant secretory factor.

Materials and Methods

Mice and Dexamethasone Treatment

Female BALB/c mice were purchased at 8 weeks of age from OrientBio (Emsung, Korea). All animals were maintained under pathogen-free conditions on a 12-h light/dark cycle with free access to food and water. All procedures were approved by the Ewha Womans University College of Medicine Animal Care and Use Committee (ESM17-0395). The mice were injected dexamethasone (2 µg per mouse per injection s.c.) on the shaved back once every 2 days (10 times in total). These dexamethasone mice were given a subcutaneous injection of dexamethasone, which had been dissolved in ethanol and diluted 1:25 in PBS immediately before injection. Normal control mice were given an injection of ethanol, which had been diluted 1:25 in PBS.

Histology and Immunohistochemistry

Skin specimens from the backs of mice were fixed in 4% formaldehyde and embedded in paraffin. Sections (5-µm thick) were mounted on slides and stained with hematoxylin and eosin (H&E) for histological evaluation. The epidermal thickness was measured on 5 slides per test group, examining 5 fields per slide using the ImageJ program.

For keratinocyte growth factor (KGF) and mast cell detection, the skin sections were stained with anti-KGF antibody (F-9; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-c-Kit antibody (H-300; Santa Cruz Biotechnology), respectively. Immunoreactivity was detected using the MULTIVIEW (mouse-HRP/rabbit-AP) IHC kit (Enzo Life Sciences, Inc., NY, USA). Tissues were subsequently counterstained with hematoxylin. Total mast cells including KGF-expressing cells were counted on the immunostained section using a magnification of ×400.

Cell Culture

The HaCaT human keratinocyte cell line was cultured in Dulbecco's modified Eagle medium (DMEM; Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS; Welgene), streptomycin (100 µg/mL), and penicillin (100 U/mL). The human HMC-1 mast cell line was kindly provided by Dr. Kim (Radiation Health Institute, Korea Hydro & Nuclear Power Co., Ltd., Seoul, Korea). HMC-1 cells were grown in Iscove's modified Dulbecco's medium (GIBCO, Grand Island, NY, USA) containing 10% FBS, streptomycin (100 µg/mL), and penicillin (100 U/mL). For dexamethasone treatment, HaCaT cells or HMC-1 cells were cultured with charcoal-stripped FBS (10%) and various concentrations of dexamethasone (0, 2.5, 25, 250, and 2,500 nM) for up to 72 h. The cell culture supernatant fractions of HMC-1 treated with 250 nM dexamethasone for 72 h were collected to treat HaCaT cells.

Reverse Transcription PCR

To analyze expression of FGF-7, the genes encoding KGF, HaCaT cells, or HMC-1 cells were treated with various concentrations of dexamethasone (0, 2.5, 25, 250, and 2,500 nM) for various periods (24, 48, and 72 h). The cells were harvested and homogenized in TRIzol (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1 µg) was transcribed into complementary DNA using a reverse transcription reagent (ELPIS-Biotech, Inc., Daejeon, Korea) according to the manufacturer's instructions. FGF-7 (123 bp) was amplified using the following primers: 5'-TCCTGCCAACTT-TGCTCTACA-3' (forward) and 5'-CAGGGCTGGAACAGTTC-ACAT-3' (reverse). The internal control gene *GAPDH* (192 bp) was amplified using the following primers: 5'-GGTAAAGTG-GATATTGTTGCCATCAATG-3' (forward) and 5'-GGAGGGA-TCTCGCTCCTGGAAGATGGTG-3' (reverse). FGF-7 expression was calculated using densitometry on UN-SCAN-IT-gel 6.1 software (Silk Scientific, Inc., Orem, UT, USA). *GAPDH* bands were used as an expression control.

Immunoblotting

HMC-1 cells treated with dexamethasone (0, 2.5, 25, 250, or 2,500 nM) for 72 h were harvested for cell lysates. Next, 20 µg of proteins were resolved by SDS-PAGE, transferred to membranes, treated in blocking agent, and incubated overnight with primary antibodies against KGF (F-9; Santa Cruz Biotechnology). After repeat washes, the membranes were incubated with the appropriate secondary antibodies and detected using an enhanced chemiluminescence reagent (Thermo Fisher Scientific, Waltham, MA, USA). Endogenous β-actin expression was detected using anti-β-actin antibody (sc-47778; Santa Cruz Biotechnology). The resulting β-actin bands were used to normalize samples using densitometry on UN-SCAN-IT-gel 6.1 software.

Cell Proliferation Assay

HaCaT cells and HMC-1 cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, CellTrace™ CFSE cell proliferation kit; Thermo Fisher Scientific) in accordance with the manufacturer's instructions. After CFSE labeling, 10⁵ cells were cultured in the presence or absence of dexamethasone at various concentrations. Simultaneously, CFSE-labeled HaCaT cells were cultured in the presence of one of the following: rhKGF (100 ng/mL; Peprotech, Rocky Hill, NJ, USA), cell culture supernatant fractions from dexamethasone (250 nM)-treated HMC-1 cells, cell culture supernates from control HMC-1 cells, or cell culture supernates from dexamethasone (250 nM)-treated HMC-1 cells containing anti-KGF blocking antibody (10 µg/mL; R&D Systems, Minneapolis, MN, USA). After 3 days, CFSE-positive cell proliferation was measured using flow cytometry and analyzed using the ModFit LT™ software (Verity Software House, Topsham, ME, USA), based on a reduction in the number of CFSE-positive cells.

Enzyme-Linked Immunosorbent Assay

For secreted KGF measurements, HMC-1 cells were treated with various concentrations of dexamethasone (0, 2.5, 25, 250, and 2,500 nM) for 72 h. Cell culture supernates were collected, and the levels of secreted KGF were determined using a human KGF ELISA kit according to the manufacturer's instructions (cat. No. DKG00; R&D Systems, Minneapolis, MN, USA).

Transepithelial Electrical Resistance

A transepithelial electrical resistance (TEER) assay was performed as reported previously. Briefly, HaCaT cells were seeded on tissue culture polycarbonate membrane filters (pore size, 0.4 µm) in 24-well Transwell plates at a seeding density of 10⁵ cells/well (Corning, Inc., Acton, MA, USA). Regular culture medium (DMEM) was added to the upper and lower chambers. Then, cell culture supernatant fractions from either dexamethasone (250 nM)-treated HMC-1 cells or from dexamethasone-treated HMC-1 cells plus anti-KGF blocking antibody (10 µg/mL; R&D Systems) were added to the lower chamber. Cells were maintained in this system for 5 days after seeding, and TEER was monitored. Electrical resistance was measured using a Millicell ERS meter (Millipore, Bedford, MA, USA; calculated as Ω cm²).

Statistics

Data are presented as means ± SEM. Statistical significance was determined by two-tailed *t* test, one-way ANOVA in conjunction with Sidak's post hoc test, two-way ANOVA in conjunction with Sidak's post hoc test, or two-way ANOVA in conjunction with Dunnett's post hoc test using GraphPad PRISM 7 software (GraphPad Software Inc., La Jolla, CA, USA). For all analyses, *p* < 0.05 was considered statistically significant.

Results

We hypothesized that GC alter skin homeostasis, particularly in the structural features. To test this hypothesis, we first examined the effect of repeated subcutaneous injections of dexamethasone, a synthetic GC, in vivo. We subcutaneously injected dexamethasone on the shaved back skin of BALB/c mice once every 2 days for 20 days. We harvested back skin tissue from control and dexamethasone-treated mice. Histological examination of the H&E-stained sections from the skin of dexamethasone-treated mice showed epidermal thickening compared with control mice (Fig. 1a), reflecting a hyperproliferative state. In the skin, keratinocytes are an important source for signals that trigger the maturation and function of mast cells. The interactive relationship between keratinocytes and mast cells suggests there may be a reverse effect of mast cells on the epidermis, particularly considering that mast cells activate under stress hormone stimulation. Thus, we investigated whether mast cells are associated with dexamethasone-induced epidermal growth in dexamethasone-treated and control mice. We found that increased epidermal growth was accompanied by increased numbers of c-kit-expressing mast cells which is visualized as red color. Further, KGF, which is a strong mitogen for epithelial cells, was detected as yellow color on mast cells from dexamethasone-treated mice. Thus, mast cells expressing both c-kit and KGF are presented as brown color (Fig. 1b). These immunostained sections revealed a

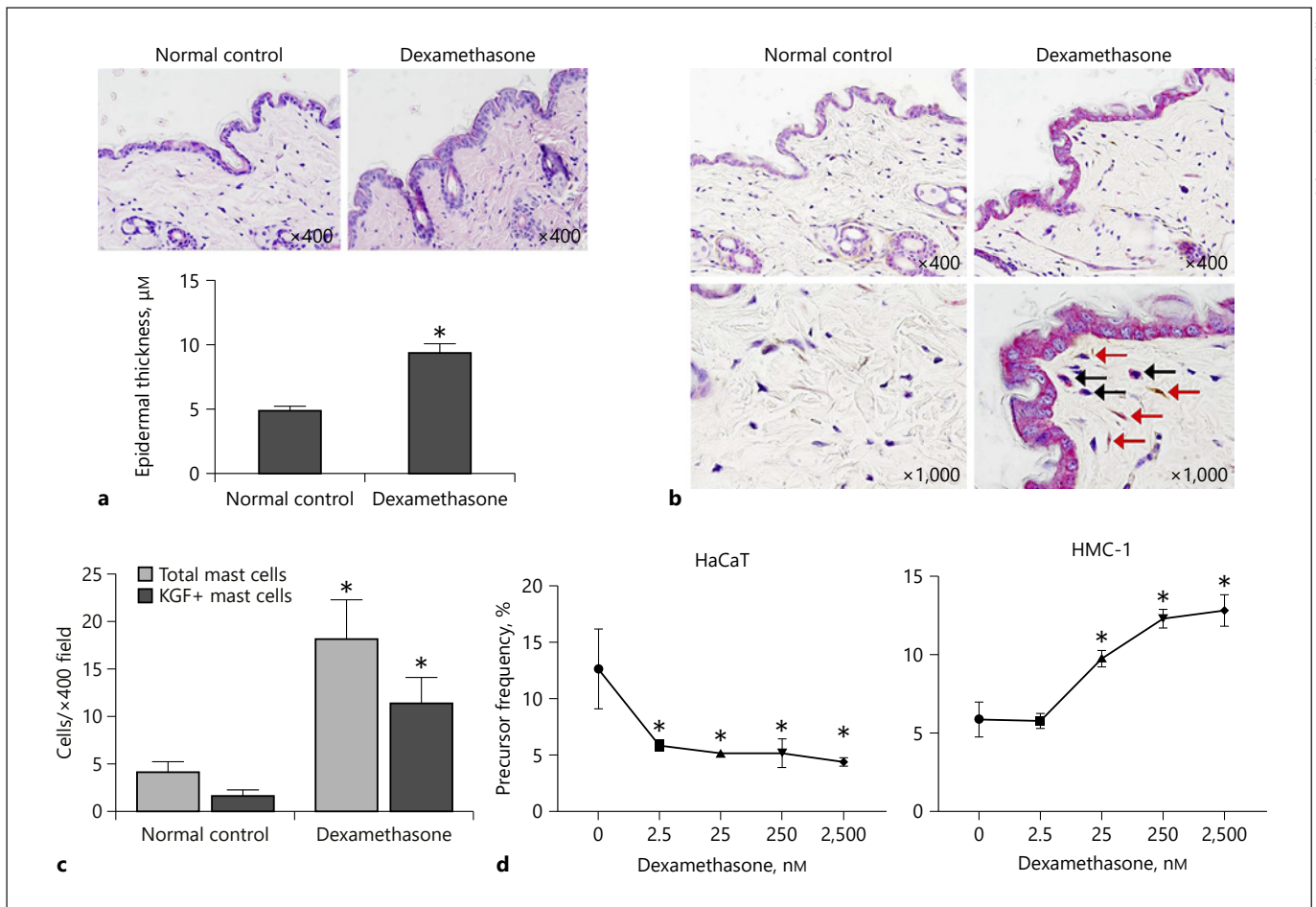


Fig. 1. Cutaneous injection of dexamethasone alters structural skin features in mice. **a** H&E-stained sections of dexamethasone-treated mice show epidermal thickening, reflecting a hyperproliferative state. No pathological findings were observed in normal control mice. Original magnification, ×400. The epidermal thickness was measured using ImageJ (lower). Data are presented as means ± SEM. The number of mice per group was 3, and the experiments were performed in triplicate ($n = 9$, $^{*}p < 0.05$ vs. control group). **b** Immunohistochemically stained sections of back skin from dexamethasone-treated and normal control mice revealed the KGF-positive mast cells in the dermis, as indicated by the arrow-head. KGF appear yellow and mast cells (c-kit) appear red. Therefore, mast cells expressing KGF are visualized as brown color. The

black arrows indicate mast cells and red arrows indicate KGF-expressing mast cells. Original magnification, ×400 (upper) and ×1,000 (lower). **c** Total mast cells and KGF-positive mast cells in immunohistochemically stained sections were counted in the ×400 field. Data are presented as means ± SEM. $^{*}p < 0.05$ vs. normal control group. **d** CFSE-labeled HaCaT cells (left) and CFSE-labeled HMC-1 (right) cells were cultured for 72 h in the presence of dexamethasone at the specified doses. The number of CFSE-positive cells was measured by flow cytometry and analyzed using the ModFit LT™ software, based on a reduction in the number of CFSE-positive cells. Data are presented as means ± SEM. $^{*}p < 0.05$ vs. dexamethasone 0 nM group. The experiments were performed in triplicate.

significant increase in total mast cells, including KGF-positive mast cells under dexamethasone treatment (Fig. 1c). This result indicates that dexamethasone increases the numbers of KGF-expressing mast cells. These changes may lead to the observed epidermal thickening. Because we showed that dexamethasone induces proliferation of the epidermis and abundance of mast cells in

vivo, we decided to examine whether dexamethasone could promote proliferation directly in each cell type. After a 3-day incubation of CFSE-labeled cells in the presence of various dexamethasone concentrations, distinct generations of proliferating cells were monitored by a flow-cytometric histogram. Based on the different peaks that represent cell generation on a flow-cytometric histo-

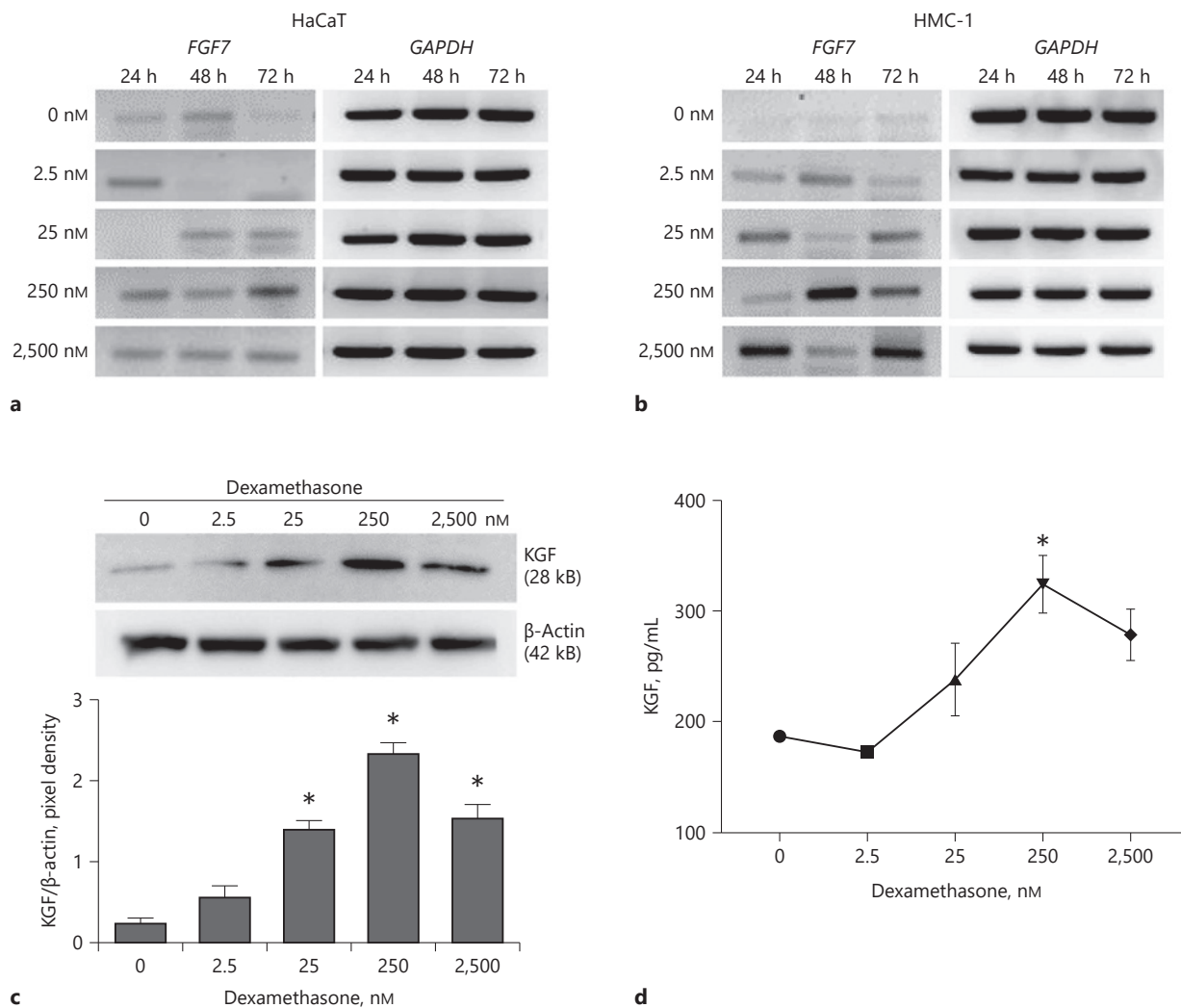


Fig. 2. Mast cells produce KGF after dexamethasone stimulation. HaCaT cells (**a**) and HMC-1 cells (**b**) were cultured with various concentrations of dexamethasone for 24, 48, or 72 h. After the indicated times, cells were harvested, and the expression of *FGF7* was detected by RT-PCR. **c** HMC-1 cells were cultured in the presence of dexamethasone at various concentrations for 72 h. The cell lysates were harvested, and the protein level of KGF was measured

by immunoblot. KGF expression was determined by densitometry, using corresponding β -actin bands for normalization. Data are presented as means $\pm$ SEM (* p < 0.05 vs. no dexamethasone). **d** Cell culture supernatant fractions from HMC-1 cells treated with dexamethasone at the specified doses for 72 h and analyzed to quantify the amount of secreted KGF by ELISA. Data are presented as means $\pm$ SEM. * p < 0.05 vs. no dexamethasone.

gram, we finally calculated the results as precursor frequency that defined the percent cells that have proliferated. Results from the CFSE assay revealed that the proliferation rate of HaCaT cells was significantly reduced by dexamethasone exposure, even at the lowest dose. However, HMC-1 cells showed an increase in proliferation af-

ter exposure to dexamethasone from concentrations of 25 nM (Fig. 1d).

Next, we investigated whether dexamethasone could induce *FGF7* (the gene encoding KGF) expression on either HaCaT cells or HMC-1 cells. HaCaT cells expressed detectable basal levels of *FGF7* without dexamethasone.

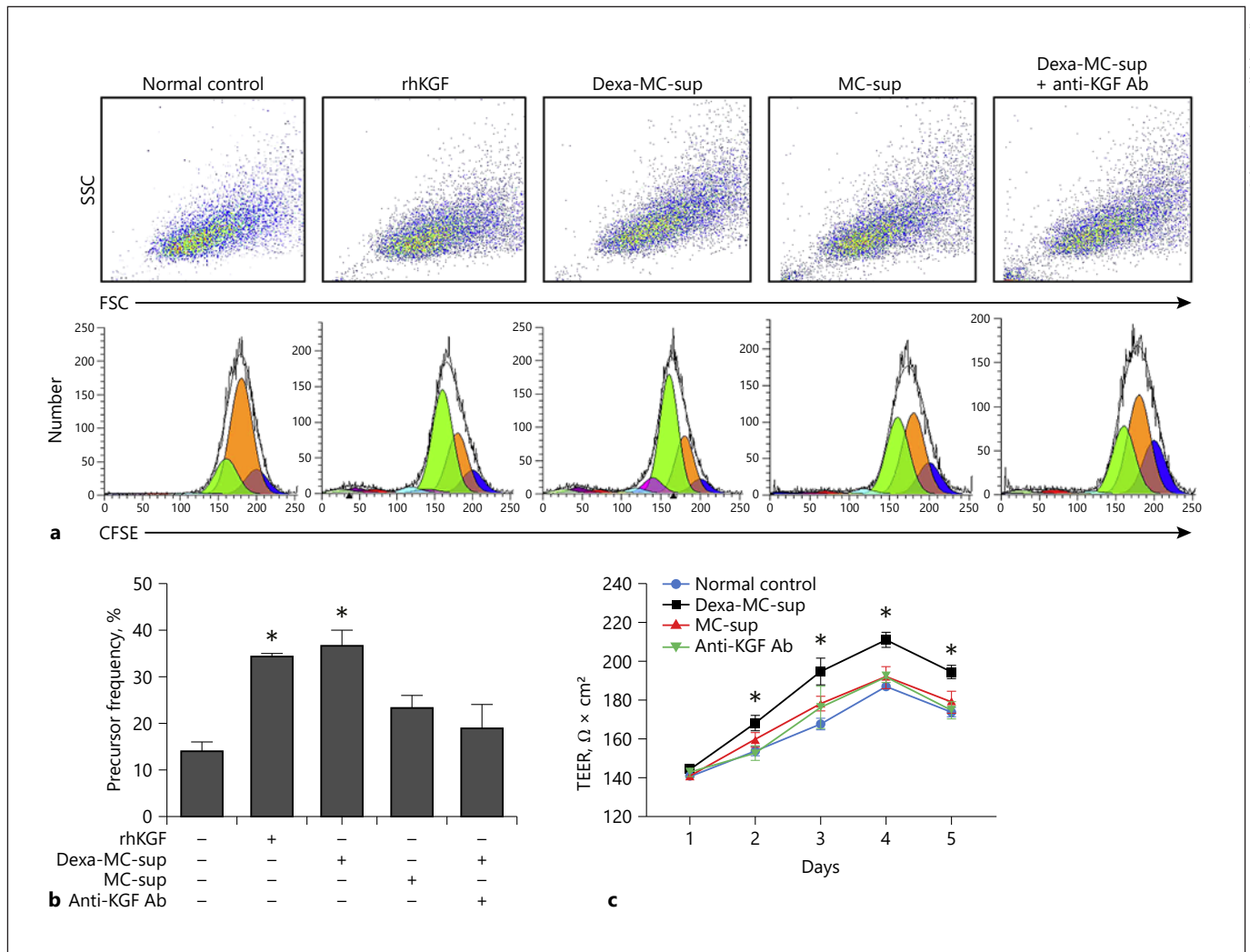


Fig. 3. Dexamethasone-treated mast cells promote keratinocyte proliferation in a KGF-dependent manner. **a** CFSE-labeled HaCaT cells were cultured in the presence of rhKGF, cell culture supernates from control HMC-1 cells (MC-sup), cell culture supernates from dexamethasone-treated HMC-1 cells (Dexa-MC-sup), or cell culture supernates from dexamethasone-treated HMC-1 cells with anti-KGF blocking antibody for 72 h. The number of CFSE-positive cells was measured by flow cytometry and analyzed using the

ModFit LT™ software, based on a reduction in the number of CFSE-positive cells. Representative dot plots (upper) and histograms (lower) are shown. The different colors in the histogram represent different generations that have proliferated. **b** Data from flow cytometry are reported as means $\pm$ SEM. * $p < 0.05$ vs. non-treated group. **c** TEER values in each experimental condition of HaCaT cells were measured for 5 days, and the data are reported as means $\pm$ SEM. * $p < 0.05$ vs. normal control group.

methasone, but there was no notable increase in expression, and they instead appear random (Fig. 2a). In HMC-1 cells, *FGF7* expression was highly induced at concentrations of 250 and 2,500 nM. But likely as HaCaT cells, the expression levels do not follow a specific pattern (Fig. 2b). When we checked the protein levels of KGF after 72 h of dexamethasone treatment, both intracellular and secreted forms of KGF were stably induced,

with the highest expression observed in the cells receiving the 250-nM dose (Fig. 2c, d).

Based on our results, we sought to determine the direct effects of mast cell-derived KGF on keratinocyte proliferation in vitro. To CFSE-labeled HaCaT cell culture, we added either the supernatant fraction from control HMC-1 cells or dexamethasone-treated HMC-1 cells and incubated them for 72 h. To test specifically the

effect of KGF on dexamethasone-treated HMC-1 supernates, anti-KGF blocking was added as an additional control. HaCaT cells were also cultured in the presence of rhKGF as a positive control. Our results suggest that dexamethasone-stimulated HMC-1 cells significantly increased proliferation of HaCaT cells; this effect was blocked when anti-KGF antibodies were present (Fig. 3a, b). Lastly, we measured TEER under the same experimental conditions for 5 days. Because TEER provides a quantitative measure of cell confluence, an increase in TEER indicates good cell monolayer health and higher confluence. As shown in Figure 3c, cell culture supernates from dexamethasone-treated HMC-1 cells significantly increased TEER over time in a KGF-dependent manner. Thus, these data indicate that dexamethasone triggers increased HaCaT cell proliferation by inducing KGF expression in mast cells.

Discussion

This study examined the influence of dexamethasone in skin homeostasis, particularly in epidermal growth. Results showed subcutaneous administration of the synthetic stress hormone dexamethasone (a GC) induces epidermal thickening and KGF expression in dermal mast cells. Further, dexamethasone directly increased the abundance of mast cells and KGF expression, but not keratinocytes, *in vitro*. Finally, dexamethasone-treated mast cells positively regulate keratinocyte proliferation in a KGF-dependent manner.

Recently, an intracutaneous HPA axis has been characterized, with a hierarchical structure similar to the central HPA axis as well as the ability to synthesize proopiomelanocortin-derived peptides and steroids [18, 19]. This signaling pathway is thought to act in an auto-/paracrine manner, fine-tuning the cutaneous stress response. It may also give feedback to the central system [18]. Moreover, recent findings that ACTH increases, but CRH decreases, the expression of the proinflammatory cytokine IL-18 in keratinocytes [20] provides another example of communication between the skin and the HPA axis. Indeed, psychosocial stressors that exceed an individual's perceived ability to cope, trigger both central and peripheral pathways. Mediators produced by these pathways combine to shape the physiological response, which affects the immune system of the skin, wound healing, barrier function, and resistance to infection [21]. Thus, stress can be a crucial initiator or trigger for relapse in chronic inflammatory skin diseases like psoriasis. In psoriasis patients, stress

modifies the distribution of leukocyte subsets, which is relevant in stress-induced exacerbation of psoriatic plaques. While the number of CD25+ regulatory T cells decreases after stress, the number of activated T cells increases, with a shift towards a Th1-derived cytokine profile. Cutaneous lymphocyte-associated antigen-positive T cells and natural killer cells also increase in the circulation during this time [22–24]. Moreover, GC strengthen certain cytokine activities at specific concentrations. For example, GC potentiate the biological action of IL-2, IFN- γ , G-CSF, and GM-CSF [25]. Acute stress activates the HPA axis, and then the effector molecule GC may potentiate the effect of the Th1 cytokine profile and contribute to aggravation of psoriatic lesions. Both acute and chronic stress can exacerbate the symptoms of psoriasis.

HPA axis hormones and their receptors, which are expressed in the skin, are known to function locally. Thus, these hormones affect the maintenance of skin homeostasis. GC are key effector molecules of the HPA axis, and they are essential for tissue homeostasis. Persistent exposure of tissues to elevated GC levels may be damaging. Murine studies have demonstrated that insomnia stress disrupts barrier function via GC-induced inhibition of epidermal lipid synthesis, which consequently impairs lamellar body formation, and decreases the size and density of corneodesmosomes [26, 27]. Subjects exhibited altered epidermal permeability barrier homeostasis at periods of increased psychological stress compared with periods of lower perceived stress [28]. Moreover, a positive psychological disposition enhances skin barrier recovery following acute psychosocial stress [29].

Because the skin senses the environment and reacts to various stressors in an effort to restore tissue homeostasis, skin resident cells, such as mast cells, could be an efficient way to mediate a stress-induced response and the consequences that occur in the skin. In fact, factors induced during stress in the brain and/or skin, such as CRH, can activate mast cells to release proinflammatory mediators. Also, CRH, sensory nerves, and the number of mast cells are increased in psoriatic lesions, producing a potential feedforward loop and promoting inflammation [30, 31]. Our results show that repeated or prolonged GC exposure specifically targets mast cells, and even *in vivo* data have shown epidermal thickening following dexamethasone treatment. Dexamethasone seemed to regulate epidermal growth indirectly, rather than through a direct action on keratinocytes. It is notable that we observed infiltration of KGF-expressing mast cells towards the epidermis in dexamethasone-

treated mice. The critical growth factor, KGF, was not detected in the epidermis after dexamethasone treatment. Indeed, dexamethasone promoted keratinocyte proliferation via mast cells in a KGF-dependent manner. Our data indicate that GC may alter the structural homeostasis in the skin, possibly via skin resident cells, such as mast cells. Considering that abnormally proliferating keratinocytes possess impaired barrier functions in certain skin diseases, additional studies are needed to measure the relevant outcomes of impaired keratinocyte proliferation in the presence of GC. In summary, this study provides several important clues regarding the mechanisms of skin homeostasis alteration, especially in individuals affected by psychological stress.

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Disclosure Statement

There are no financial conflicts of interest.

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