Increased Hyaluronan Production and Decreased E-Cadherin Expression by Cytokine-Stimulated Keratinocytes Lead to Spongiosis Formation

Tomoyuki Ohtani[1], Ai Memezawa[1], Ryuhei Okuyama[1], Tetsuya Sayo[2], Yoshinori Sugiyama[2], Shintaro Inoue[2] and Setsuya Aiba[1]

The pathogenesis of spongiosis, which is a well-known hallmark of acute eczema, is not fully understood. We sought to clarify the mechanism for the influx of tissue fluid into the epidermis and the loss of cohesion between keratinocytes in acute eczema that result in spongiosis. We first demonstrated increased intercellular accumulation of hyaluronan (HA) in the spongiotic epidermis by immunochemical staining using hyaluronic-acid-binding protein (HABP) and augmented hyaluronan synthase 3 (*HAS3*) mRNA expression by spongiotic keratinocytes using *in situ* hybridization. We also showed that the epidermis where the intercellular space was strongly stained with HABP showed weaker expression of membrane E-cadherin. Next, we demonstrated—by a sandwich assay using HABP, real-time PCR, and flow cytometry—that, among various cytokines, only IL-4, IL-13, and IFN-γ increased HA production, enhanced *HAS3* mRNA expression, and decreased membrane E-cadherin expression by normal human epidermal keratinocytes in both low- and high-Ca media. Finally, we demonstrated IL-4, IL-13, their combination, and IFN-γ could induce intercellular space widening of the epidermis with increased HA accumulation and decreased E-cadherin expression in the organotypic culture. These results suggest that the augmented production of HA and the decreased E-cadherin expression by keratinocytes stimulated with IL-4/IL-13 or IFN-γ cause spongiosis in acute eczema.

Journal of Investigative Dermatology (2009) **129,** 1412–1420; doi:10.1038/jid.2008.394; published online 1 January 2009

INTRODUCTION

Spongiosis is a characteristic histopathological feature in acute eczema (Holden and Berth-Jones, 2004). However, studies on its pathogenesis are limited. It has been believed that spongiosis is caused by the secondary loss of cohesion between epidermal cells due to the influx of tissue fluid into the epidermis. Serous exudate extends from the dermis into the intercellular compartment of the epidermis; as it expands, epidermal cells remain in contact with each other only at the sites of desmosomes, acquiring a stellate appearance and giving the epidermis a sponge-like morphology (Wolff *et al.*, 2003).

Recently, Trautmann *et al.* (2000, 2001) have demonstrated that in allergic contact dermatitis (ACD), IFN-γ-producing T cells trigger Fas-induced apoptosis of keratinocytes and that apoptotic keratinocytes have decreased E-cadherin expression that results in cleavage of E-cadherin, while maintaining desmosomal cadherins. When we consider that E-cadherin and desmosomal cadherin, both of which are Ca^{2+}-dependent transmembrane glycoproteins, constitute adherens junction and desmosome, respectively (reviewed by Jensen and Wheelock, 1996), these observations can partly explain the mechanism of spongiosis formation in ACD. However, the mechanism for the influx of tissue fluid into the epidermis has not yet been clarified.

Moreover, although, classically, ACD in mice was regarded as a Th1 cytotoxic cell-mediated immune response (Cher and Mosmann, 1987; Fong and Mosmann, 1989; Grabbe and Schwarz, 1998), recent evidence indicates that the induction of contact hypersensitivity responses is dependent on the activity of Th2 cells (Dieli *et al.*, 1994; Salerno *et al.*, 1995; Yokozeki *et al.*, 2000; Takeshita *et al.*, 2004). Moreover, Neis *et al.* (2006) have reported that the large majority of biopsy specimens of ACD in human displayed high expression levels (more than fivefold median increase) of IL-4 and IL-13, whereas a more than fivefold increase in IFN-γ mRNA levels was only detected in a limited number of patients. These data strongly suggest that IL-4 and/or IL-13 are more crucial than IFN-γ in the pathogenesis of ACD in both human and mice. Therefore, IFN-γ alone seems to be insufficient to explain the pathogenesis of spongiosis.

[1]*Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai, Japan and* [2]*Kanebo Cosmetics Inc., Odawara, Japan*

Correspondence: Dr Setsuya Aiba, Department of Dermatology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aobaku, Sendai 980-8574, Japan. E-mail: aiba@mail.tains.tohoku.ac.jp

Abbreviations: ACD, allergic contact dermatitis; AD, atopic dermatitis; DIG, digoxigenin; HA, hyaluronan; HABP, hyaluronic-acid-binding protein; ISH, in situ *hybridization; Mapkbp1, mitogen-activated protein-kinase-binding protein 1; NHEK, normal human epidermal keratinocyte*

Received 17 April 2008; revised 2 September 2008; accepted 17 October 2008; published online 1 January 2009

Hyaluronan (HA) is a high-molecular-weight linear glycosaminoglycan consisting of alternating glucuronic acid and *N*-acetylglucosamine residues (Fraser *et al.*, 1997). Although HA is a well-known component of connective tissues, for example, the cartilage of joints and the dermis of skin, its importance in the skin epidermis is now gaining attention (Maytin *et al.*, 2004). In human epidermis, Tammi *et al.* (1988) and Wells *et al.* (1991) have reported, using hyaluronic-acid-binding protein (HABP) that HA is localized in the matrix between keratinocytes in the middle and upper parts of the spinous layer. Moreover, we have recently reported that even the normal stratum corneum contains HA supplied by keratinocytes (Sakai *et al.*, 2000).

The carboxyl groups of HA are fully ionized at extracellular pH. Its osmotic activity is nonideal and is disproportionately high in relation to its molecular weight. In addition, the molecular meshwork of HA causes steric exclusion of other macromolecules. For these reasons, HA is capable of having profound effects on the distribution and movement of water and plays a major part in water homeostasis (Laurent and Fraser, 1992; Fraser *et al.*, 1997).

To clarify the mechanism for the movement of water in spongiosis, in this study we first compared HA accumulation in the epidermis in acute eczema showing spongiosis and other inflammatory skin diseases using histochemical staining with HABP. We then conducted *in situ* hybridization (ISH) to demonstrate hyaluronan synthase 3 (*HAS3*) mRNA expression by spongiotic keratinocytes. Next, we examined whether HA accumulation was localized in the epidermis with decreased E-cadherin expression, as decreased E-cadherin expression in spongiotic epidermis has been reported (Trautmann *et al.*, 2001). To explore the mechanism by which spongiotic epidermis increases the production of HA, we investigated *HAS1* mRNA, *HAS2* mRNA, and *HAS3* mRNA expression and HA production by normal human epidermal keratinocytes (NHEK) stimulated with various cytokines including IL-4 and IL-13. In addition, we examined the regulation of membrane E-cadherin expression by keratinocytes treated with IL-4, IL-13, or IFN-γ. Finally, we confirmed the role of IL-4, IL-13, and IFN-γ in the pathogenesis of spongiosis using an organotypic culture.

RESULTS

Increased HA in the intercellular space of spongiosis

We first examined the localization of HA in the various tissue samples by histochemical staining with HABP. Representative tissue samples stained with hematoxylin–eosin and HABP are shown in Figure 1. As reported previously (Tammi *et al.*, 1988; Wells *et al.*, 1991), HABP staining was mainly localized in the middle and upper parts of the spinous layer of normal skin, although the staining was not necessarily intense (Figure 1b). In contrast, strong HABP staining from the basal layer to the upper spinous layer was present in all of the specimens of acute eczema (Figure 1d). More intense staining was localized to the regions exhibiting widened intercellular spaces, that is, spongiosis (Figure 1e). In our study, we included ACD and dysidrotic eczema as acute eczema, both of which showed obvious spongiosis histologically. Between these two disorders, we did not detect any significant difference in HABP staining. To examine whether strong HABP staining was a common feature of the histopathological changes accompanying the widened intercellular spaces in the epidermis, we examined the localization of HA in acantholytic epidermis of pemphigus vulgaris (Figure 1f). In contrast to acute eczema, significant HABP staining was not detected in the acantholytic clefts of any cases or in the intercellular spaces surrounding those clefts, suggesting that HA is not deposited in the intercellular space of the epidermis due to the lack of epidermal cohesion. When we examined the lesional skin of psoriasis and parapsoriasis en plaque as control skin disorders accompanied by lymphocytic exocytosis, HABP staining was more intense in the epidermis with either disease than in normal epidermis (Figures 1g and h). However, the staining intensity was far weaker than that in the spongiotic epidermis.

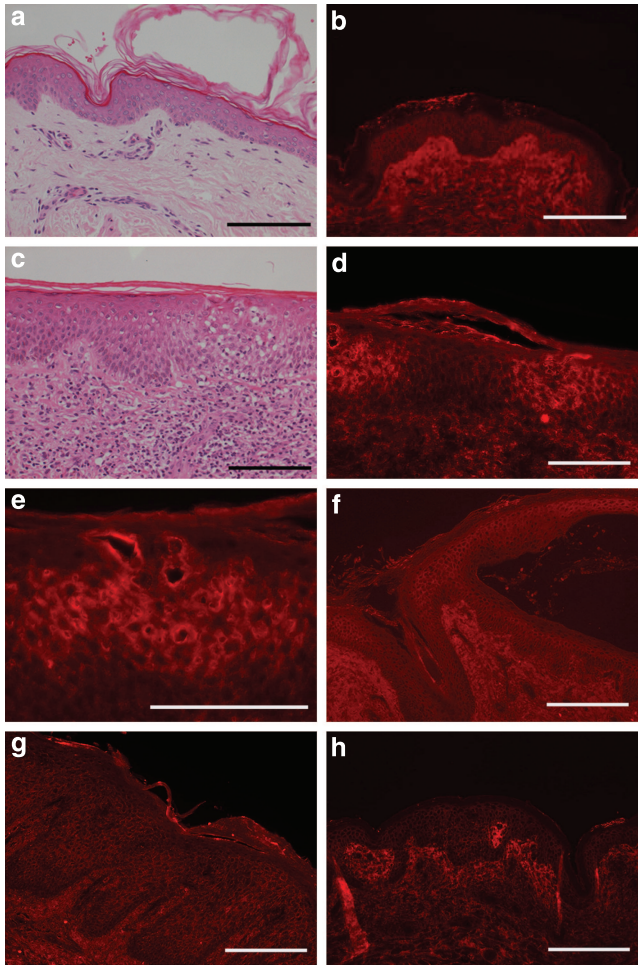

Figure 1. The accumulation of HA in various skin disorders. Deparaffinized 4 μm sections of normal skin (**a** and **b**) and various skin disorders including acute eczema (**c**, **d**, and **e**), pemphigus vulgaris (**f**), psoriasis vulgaris (**g**), and parapsoriasis en plaque (**h**) were stained with hematoxylin–eosin (**a** and **c**) and with biotinylated HABP and streptavidin-Texas Red (**b**, **d**, **e**, **f**, **g**, and **h**). Original magnification was × 100 except for (**e**) (× 200), bars = 0.1 mm (**a**–**h**).

Augmented *HAS3* mRNA expression by keratinocytes in spongiotic lesions

Recently, we reported that cultured human keratinocytes expressed *HAS1* and *HAS3* mRNAs, whereas *HAS2* mRNA was undetectable or detected as a trace band by northern blot analysis using total RNA or poly(A)+ RNA. In addition, only *HAS3* mRNA was dramatically upregulated in keratinocytes treated with IFN-γ and downregulated by TGF-β (Sayo *et al.*, 2002). Therefore, in this study, we evaluated the expression of *HAS3* mRNA in the epidermis of acute eczema and normal skin by *in situ* mRNA hybridization (Sayo *et al.*, 2002). Using a digoxigenin (DIG)-labeled *HAS3* anti-sense RNA probe, we found that *HAS3* mRNA was expressed in the basal to granular layers in normal skin (Figure 2a). Interestingly, *HAS3* mRNA was more intensely expressed in the middle part of the spongiotic epidermis (Figure 2c). No significant signals were detected by the DIG-labeled mitogen-activated protein-kinase-binding protein 1 (Mapkbp1) sense RNA probe (Figures 2b and d), indicating that the signals derived from the DIG-labeled *HAS3* anti-sense RNA probe were specific.

Spongiotic epidermis increased HA in the intercellular space and downregulated membrane E-cadherin expression

Recently, Trautmann *et al.* (2000, 2001) reported that the spongiotic epidermis of atopic dermatitis (AD) as well as that of ACD showed a reduction in keratinocyte membrane E-cadherin, but not in desmosomal cadherins. Therefore, we next examined the correlation between HABP staining and E-cadherin expression in the lesional skin of acute eczema. When compared with normal skin (Figure 3a–c), even the epidermis of acute eczema, which did not show significant widening of the intercellular space, that is, spongiosis, had decreased E-cadherin expression (Figure 3d–f). However, the epidermis where the intercellular space was strongly stained with HABP showed weaker expression of membrane E-cadherin. These findings demonstrate that the augmented HA production correlates with the downregulation of membrane E-cadherin expression, suggesting a possible link between these phenomena.

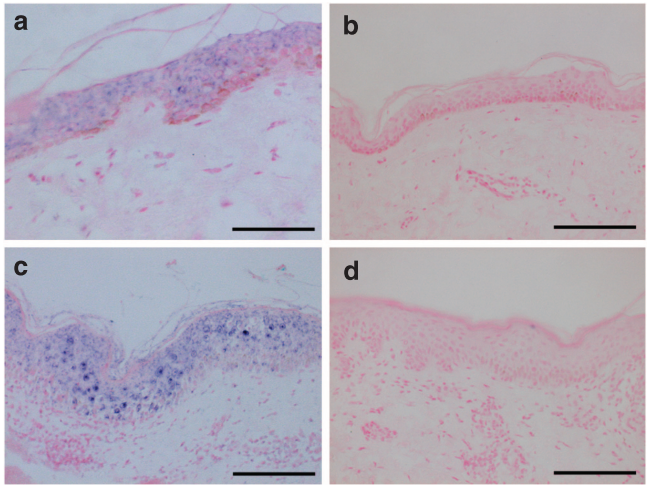

Figure 2. *HAS3* mRNA expression in normal skin and acute eczema by *in situ* hybridization. *HAS3* mRNA expression in normal skin (**a** and **b**) and acute eczema (**c** and **d**) was examined by ISH using a DIG-labeled anti-sense RNA for human *HAS3* mRNA (**a** and **c**) and DIG-labeled mouse Mapkbp1 sense RNA (**b** and **d**). Original magnification was × 100, bars = 0.1 mm (**a**–**d**).

Augmented *HAS3* mRNA expression by keratinocytes stimulated with IL-4, IL-13, and IFN-γ

To elucidate the mechanism by which keratinocytes augment *HAS3* mRNA expression, we examined *HAS3* mRNA expression by NHEK in low-Ca medium 24 hours after the stimulation with three different concentrations, 10, 30, and 100 ng ml^{-1}, of various cytokines. Among these cytokines, both IL-4 and IL-13 in addition to IFN-γ, which we previously reported as augmenting *HAS3* mRNA expression (Sayo *et al.*, 2002), stimulated *HAS3* mRNA expression. None of the other cytokines—TNF-α, IL-1β, IL-5, IL-6, IL-15, IL-17A, IL-18, or IFN-α—augmented *HAS3* mRNA, even at the highest concentration (Figure 4a and b). Lower concentrations of TNF-α, IL-1β, IL-5, IL-6, IL-15, IL-17A, and IL-18 also failed to stimulate *HAS3* mRNA expression (data not shown). Although the effects of the cytokines on *HAS3* mRNA expression by NHEK were consistent, the magnitude of *HAS3* mRNA induction varied depending on the cell lot. To further clarify the augmentation of *HAS3* mRNA expression by keratinocytes stimulated with IL-4, IL-13, and IFN-γ, we performed three additional experiments and calculated the percent augmentation of *HAS3* mRNA expression by keratinocytes stimulated with three different concentrations of these cytokines and statistically analyzed the effects (Figure 4c). In addition to IFN-γ, 10–100 ng ml^{-1} of IL-4 and 100 ng ml^{-1} of IL-13 significantly augmented the *HAS3* mRNA expression by keratinocytes, although the augmentation was smaller than that with IFN-γ. When we examined *HAS3* mRNA expression by NHEK cultured in high-Ca medium, identical results with those from NHEK cultured in low-Ca medium were obtained. Only IL-4, IL-13, and IFN-γ augmented *HAS3* mRNA expression (Figure S1a).

In our previous studies (Sayo *et al.*, 2002), we demonstrated that cultured NHEK expressed both *HAS1* and *HAS3* mRNA, but not *HAS2* mRNA and that only *HAS3* mRNA was regulated by IFN-γ and TGF-β. In these studies, however, we examined *HAS1*, *HAS2*, and *HAS3* mRNA expression only in low-Ca medium. Therefore, in the current study, we examined *HAS1* and *HAS2* mRNA expression by NHEK cultured in both low- and high-Ca media. In both low- and high-Ca media, NHEK did not express detectable *HAS2* mRNA after any cytokine treatment (data not shown). On the other hand, NHEK cultured in both low- and high-Ca media expressed *HAS1* mRNA, although no cytokines affected their expression significantly (Figures S1b and Sc).

Augmented HA production by keratinocytes stimulated with IL-4, IL-13, or IFN-γ

As IL-4, IL-13, and IFN-γ significantly augmented *HAS3* mRNA expression, we measured HA in the culture supernatants of NHEK treated for 24 hours with IL-4 (10, 30, and 100 ng ml^{-1}), IL-13 (10, 30, and 100 ng ml^{-1}), and IFN-γ (25 ng ml^{-1}). Initially, NHEK produced substantial amounts of HA without any stimulation. Consistent with the mRNA expression results,

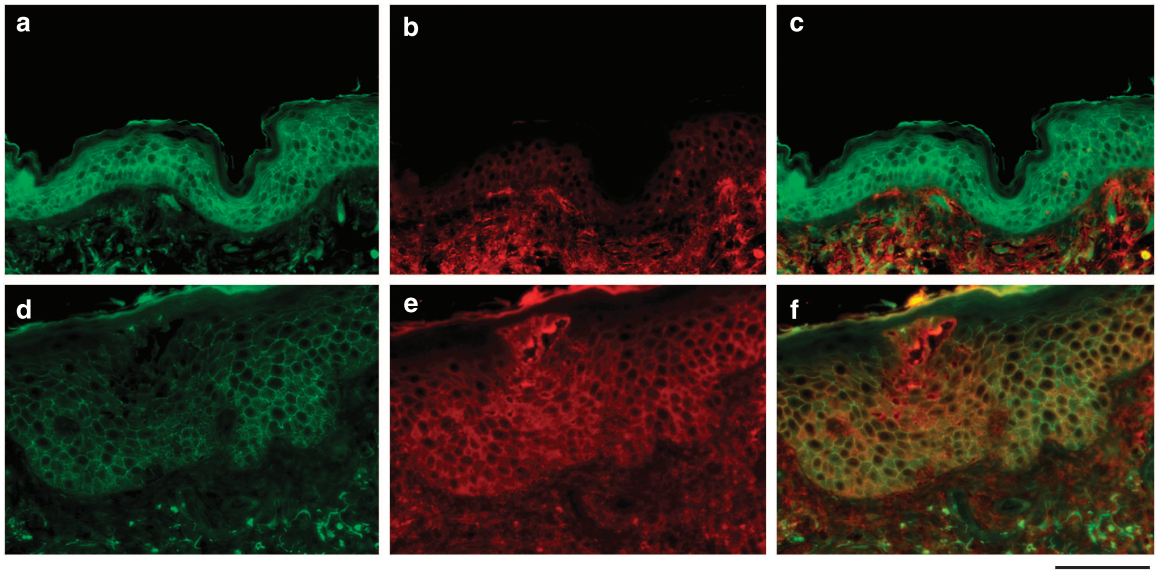

Figure 3. HA accumulation and E-cadherin expression in spongiotic epidermis. HA accumulation and E-cadherin expression were determined by double fluorescence staining using a combination of biotinylated HABP and streptavidin-Texas Red and anti-human E-cadherin Ab and FITC-labeled goat anti-mouse Ig. (**a**, **b**, and **c**): normal skin; (**d**, **e**, and **f**): acute eczema; (**a** and **d**): anti-E-cadherin Ab; (**b** and **e**): HABP; (**c** and **f**): merged. Original magnification $\times 100$, bar = 0.1 mm (**a**–**f**).

NHEK stimulated with 10–100 ng ml^{-1} of IL-4, 30 ng ml^{-1}, and 100 ng ml^{-1} of IL-13, or 25 ng ml^{-1} of IFN-γ significantly augmented the production of HA (Figure 5).

Suppressed E-cadherin expression by keratinocytes treated with either IL-4 or IL-13

As IL-4 or IL-13 increased *HAS3* mRNA expression and HA production, we next examined whether these cytokines affected the E-cadherin expression by keratinocytes. Fujii-Maeda *et al*. (2004)reported that IL-4/IL-13 downregulated E-cadherin expression by a spontaneously transformed keratinocyte cell line, HaCaT, and by NHEK. However, the suppression of E-cadherin expression by NHEK treated with IL-4 or IL-13 was not statistically significant. As they examined the effects of these cytokines in NHEK cultured in low-Ca medium, we examined their effects on NHEK that were cultured in low- or high-Ca medium. Consistent with the previous studies using confocal scanning microscope that demonstrated stronger expression of E-cadherin by NHEK cultured in high-Ca medium than those in low-Ca medium (Fujii-Maeda *et al*., 2004), our flow cytometry also clearly demonstrated that high-Ca medium significantly augmented E-cadherin expression by NHEK. Interestingly, only keratinocytes cultured in high-Ca medium significantly downregulated E-cadherin expression in the presence of IL-4 or IL-13. In this experiment, IFN-γ also downregulated E-cadherin expression in the keratinocytes cultured in high-Ca medium, but the difference was not statistically significant (Figure 6).

Induction of spongiosis with increased HA accumulation and decreased E-cadherin expression in the organotypic culture treated with cytokines

To further confirm the importance of IL-4, IL-13, or IFN-γ in the pathogenesis of spongiosis, we treated the three-dimensional EpiDerm culture (EPI-200) with these three cytokines (Figure 7). Interestingly, IL-4, IL-13, their combination, or IFN-γ clearly induced intercellular space widening of the epidermis with visible intercellular bridges. Together with spongiosis formation, these cytokines increased HA accumulation in the intercellular space of the epidermis and decreased epidermal E-cadherin expression. Among these treatments, the combination of IL-4 and IL-13 showed the most prominent effect.

DISCUSSION

In this study, we first demonstrated HA accumulation in the intercellular space of the epidermis in specimens of acute eczema and its more abundant accumulation in the spongiotic epidermis. Wells *et al*. (1991) have reported diffuse HABP staining in epidermis with positive patch-test reactions. However, they did not report a correlation between HA accumulation and spongiotic changes, probably because their specimens were obtained from lesions with more intense inflammation than our specimens. Our histochemical staining demonstrated that the lesional epidermis of pemphigus vulgaris, which also exhibited intercellular space widening caused by acantholysis, did not have increased HA accumulation in the intercellular spaces or in the clefts of the epidermis. This indicated that increased HA in the intercellular spaces of spongiotic epidermis was not a simple consequence of the loss of epidermal cell cohesion. Furthermore, the examination of other skin disorders with exocytosis of lymphocytes, for example, psoriasis vulgaris and parapsoriasis en plaque, indicated that the significantly high HA accumulation observed in the spongiotic epidermis was not a general phenomenon of skin disorders characterized by lymphocyte exocytosis, although psoriasis vulgaris and parapsoriasis en

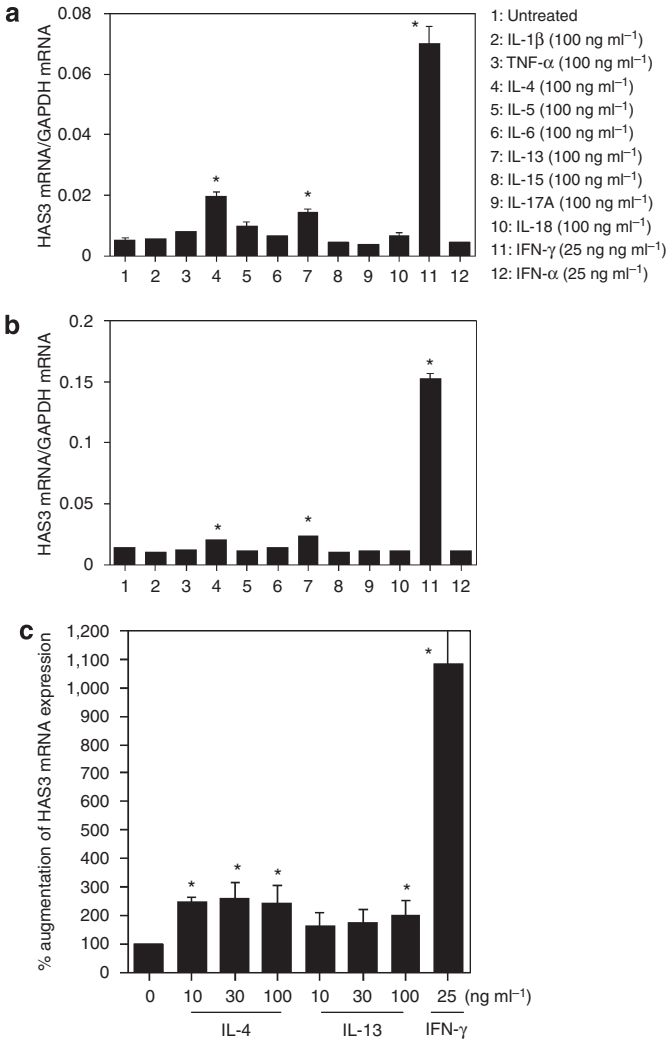

Figure 4. *HAS3* mRNA expression by NHEK stimulated with various cytokines. After 24 hours of stimulation with various cytokines, the ratio of *HAS3* mRNA/GAPDH mRNA of NHEK cultured in low-Ca medium was calculated. The mean ratio ± SD is shown for each cytokine. (**a** and **b**) Representative data from two of four independent experiments are shown. **P*<0.05 by the unpaired *t*-test. (**c**) Three further independent experiments were performed and the percent augmentation of *HAS3* mRNA expression was calculated. The mean ± SEM of the percent augmentation is shown. **P*<0.05 by the paired *t*-test.

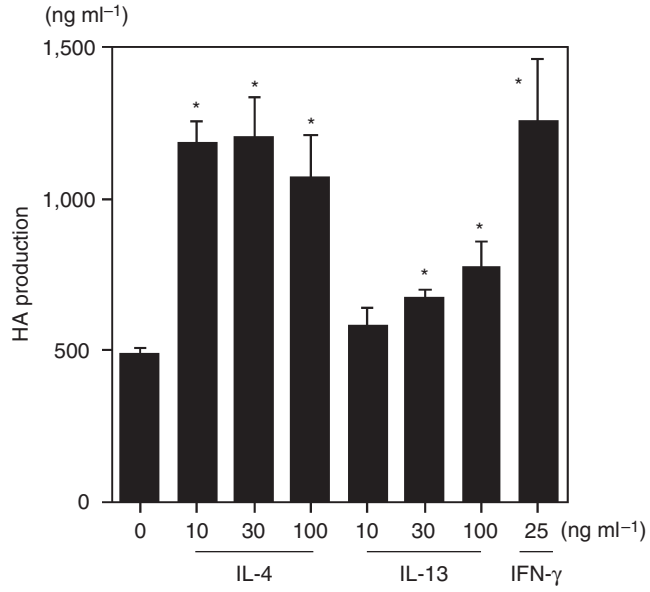

Figure 5. HA production by NHEK stimulated with IL-4, IL-13, or IFN-γ. HA in the culture supernatants of NHEK treated for 24 hours with IL-4 (10, 30, or 100 ng ml^{-1}), IL-13 (10, 30, or 100 ng ml^{-1}), or IFN-γ (25 ng ml^{-1}) was measured by the sandwich assay using HABP. The data are means ± SD of three independent experiments. **P*<0.05 by the unpaired Student's *t*-test.

plaque did show increased HA accumulation in the epidermis.

HA is synthesized by hyaluronan synthase plasma membrane proteins encoded by the corresponding members of a multi-gene family: *HAS1*, *-2*, and *–3* (Sayo *et al.*, 2002; Monslow *et al.*, 2003). We have reported that human keratinocytes express only *HAS1* and *HAS3* (Sayo *et al.*, 2002), whereas all hyaluronan synthase isoenzymes are expressed in the monolayers of rat keratinocytes and at least *HAS1* and *HAS2* are expressed in mouse keratinocytes (reviewed by Pasonen-Seppänen *et al.*, 2003). In addition, only *HAS3* mRNA expression is regulated by cytokines such as IFN-γ and TGF-β in human keratinocytes (Sayo *et al.*, 2002). Because, in our previous studies, we focused on the effects of IL-1α, TNF-α, IL-8, IL-10, IFN-γ, and TGF-β and examined their effects on NHEK cultured in only low-Ca medium, in this study, we investigated the effects of cytokines including IL-4, IL-5, IL-6, IL-13, IL-15, IL17A, and IL-18 on *HAS1*, *HAS2*, and *HAS3* mRNA expression by NHEK cultured in both low- and high-Ca media. These studies confirmed that only *HAS3* mRNA was upregulated by IL-4, IL-13, and IFN-γ irrespective of Ca concentration. Moreover, HA production was also augmented by these three cytokines. Unexpectedly, although IFN-γ augmented *HAS3* mRNA more vigorously than either IL-4 or IL-13, HA production was not necessarily increased more abundantly by IFN-γ than IL-4 or IL-13. This may be due to the promoting effects of IFN-γ on keratinocyte differentiation, which suppresses the growth of keratinocytes (Saunders and Jetten, 1994).

On the basis of these *in vitro* studies, we focused on *HAS3* mRNA expression in acute eczema. Consistent with the increased HA concentration, keratinocytes in the spongiotic epidermis showed increased *HAS3* mRNA expression. In our ISH, we used a DIG-labeled mouse Mapkbp1 sense RNA probe. Initially, we attempted to use a DIG-labeled *HAS3* sense probe; however, northern blot analysis demonstrated that the DIG-labeled *HAS3* sense probe reacted with an unknown mRNA, other than *HAS3*, extracted from cultured human keratinocytes and fibroblasts. Therefore, we used a DIG-labeled mouse Mapkbp1 sense RNA probe of a length and GC content similar to those of the *HAS3* anti-sense probe, which did not show any cross-reactivity with mRNA from human tissue (data not shown). Our *in vitro* studies using NHEK suggested the major role of *HAS3* in HA production by keratinocytes in the spongiotic epidermis because NHEK did not express *HAS2* mRNA in either low- or

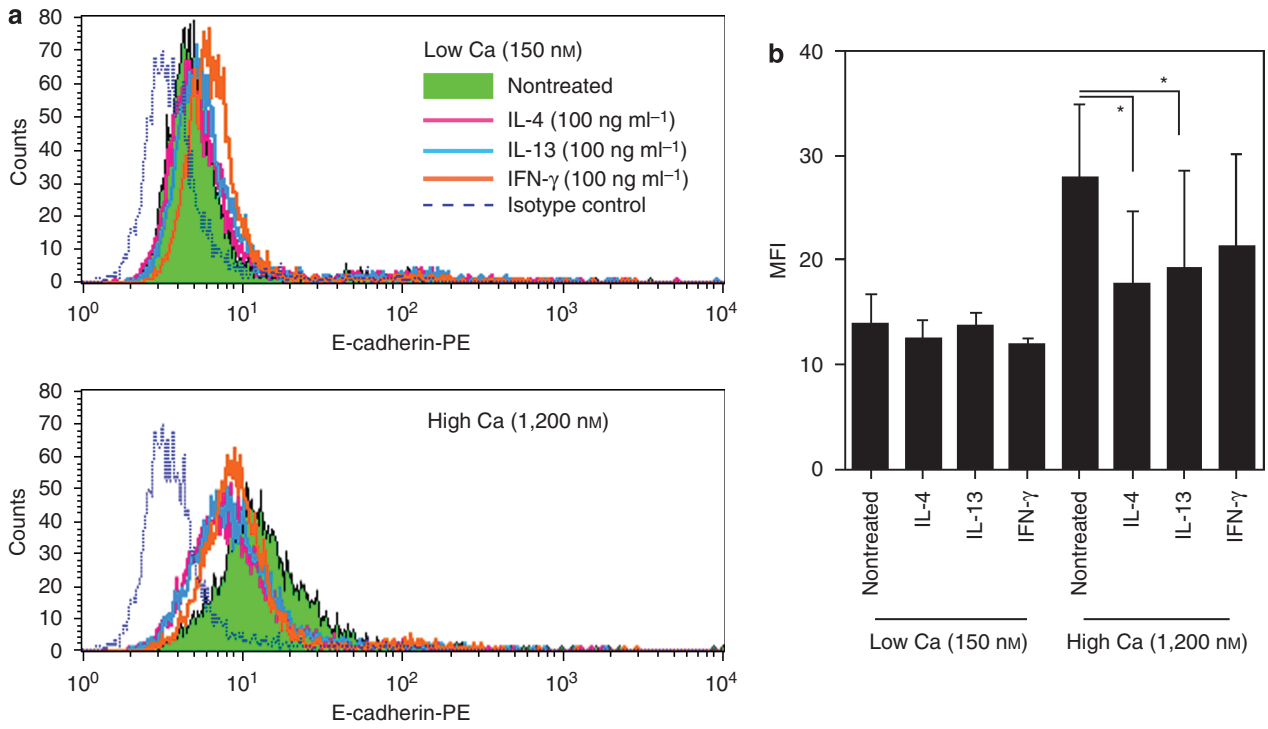

Figure 6. E-cadherin expression by NHEK stimulated with IL-4, IL-13, or IFN-γ. NHEK were cultured with IL-4, IL-13, or IFNγ in both low calcium (0.15 mM Ca^{2+}) and high calcium (1.2 mM Ca^{2+}) conditions for 72 hours and the expression of E-cadherin was examined by flow cytometry. (**a**) Representative data from three independent experiments are shown. (**b**) The summarized data from three independent experiments are shown. The data are means ± SEM of three independent experiments. *$P<0.05$ by the paired *t*-test.

high Ca-medium, whereas *HAS1* mRNA expression was not affected by any cytokines. However, it is necessary to examine *HAS1* and *HAS2* mRNA expression in the spongiotic epidermis by ISH to obtain a final conclusion. This issue is currently under the investigation.

Next, we examined the localization of HA accumulation and E-cadherin expression in acute eczema, because Trautmann *et al.* (2000, 2001) have demonstrated decreased E-cadherin expression and maintained desmosomal cadherin expression in ACD. Interestingly, in all the examined cases with acute eczema, the epidermis in which the intercellular spaces were strongly stained with HABP showed weaker membrane E-cadherin expression.

Although Trautmann *et al.* (2000, 2001) have demonstrated the role of IFN-γ in the pathogenesis of spongiosis, recent evidence indicates the crucial role of Th2 cells in the pathogenesis of ACD (Salerno *et al.*, 1995; Asherson *et al.*, 1996; Yokozeki *et al.*, 2000; Takeshita *et al.*, 2004; Neis *et al.*, 2006). Therefore, it is conceivable that IL-4 and/or IL-13 are involved in the pathogenesis of spongiosis. Indeed, our current study clearly demonstrated that IL-4/IL-13 down-regulated membrane E-cadherin expression by NHEK cultured in high-Ca medium.

On the basis of these observations, we propose the following mechanism for the spongiosis formation in acute eczema. In acute eczema, IL-4/IL-13-producing T cells and/or IFN-γ-producing T cells infiltrate into the epidermis. Both IL-4/IL-13 and IFN-γ induce decreased membrane E-cadherin expression by keratinocytes possibly through different mechanisms, while they increase *HAS3* mRNA expression and HA production in keratinocytes. Increased HA in the intercellular space of the epidermis induces the movement of water into the epidermis by osmosis and its molecular meshwork (Laurent and Fraser, 1992; Fraser *et al.*, 1997). The resulting increased hydrostatic pressure widens the intercellular space, elongating and distorting portions of the epidermal cell surfaces that lose their E-cadherin adhesion but retain desmosomal adhesion. This suggested mechanism was strongly supported by our observation that IL-4, IL-13, their combination, and IFN-γ clearly induced intercellular space widening in the epidermis with augmented HA accumulation and decreased E-cadherin expression in the oragnotypic culture.

Thus far, the biological significance of spongiosis has not been considered extensively. Rather, it has been understood as a simple consequence of epidermal damage by the haptens themselves and by T-cell-mediated cytotoxicity. However, our study shows that keratinocytes stimulated by IFN-γ and IL-4/IL-13 actively synthesize HA, which suggests that spongiosis is an adaptive process by keratinocytes to dilute noxious chemicals by increasing water in the intercellular space of the epidermis.

MATERIALS AND METHODS

Reagents

Recombinant human TNF-α, IL-1β, IL-4, IL-5, IL-6, IL-13, IL-15, IL-17A, IL-18, IFN-α, and IFN-γ were purchased from Peprotech EC Ltd, London, UK.

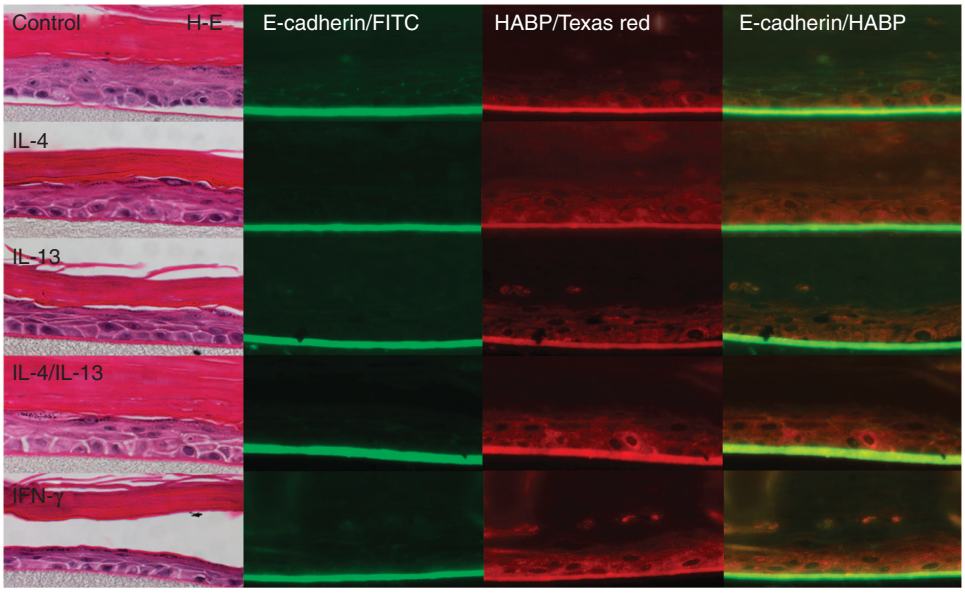

Figure 7. The histological change, HA accumulation, and E-cadherin expression in the organotypic culture treated with cytokines. The EPI-200 cultures were grown at the air–liquid interface and stimulated with 100 ng ml^{-1} of IL-4, IL-13, their combination, or 25 ng ml^{-1} of IFN-γ for 4 days. After culture, they were processed for hematoxylin–eosin staining, immunofluorescence staining for E-cadherin, or immunochemical staining using HABP. They are representative data from triplicate cultures. Bar = 0.1 mm.

Tissue samples

Following ethical approval of Tohoku University Graduate School of Medicine, Sendai, Japan, and adhering to the Declaration of Helsinki Principles, we analyzed formalin-fixed paraffin-embedded lesional skin specimens from 28 patients with acute eczema showing spongiosis histologically (17, contact dermatitis; 11, dysidrotic eczema), 12 with psoriasis, 6 with pemphigus vulgaris, and 10 with parapsoriasis en plaque, and 10 normal skin specimens from the Department of Dermatology, Tohoku University Graduate School of Medicine. All the subjects gave informed consent before the examinations.

Cell culture

NHEK from neonatal foreskin (Kurabo, Osaka, Japan) were cultured in a fully supplemented (10 μg ml^{-1} bovine insulin, 0.5 μg ml^{-1} hydrocortisone, 0.1 ng ml^{-1} human epidermal growth factor, 0.4% vol/vol bovine pituitary extract, 50 μg ml^{-1} gentamicin, 50 ng ml^{-1} amphotericin B), low calcium (0.15 mM Ca^{2+}), serum-free keratinocyte growth medium (HuMedia-KG2; Kurabo). They were used at the second passage. For 24 hours before and during the following experiments, the cells were cultured in medium without insulin, hydrocortisone, human epidermal growth factor, and bovine pituitary extract. To examine the effects of cytokines on HA production and *HAS3* mRNA expression by NHEK, TNF-α, IL-1β, IL-4, IL-5, IL-6, IL-13, IL-15, IL-17A, and IL-18 (10, 30, and 100 ng ml^{-1}), IFN-α (25 ng ml^{-1}), and IFN-γ (25 ng ml^{-1}) were added to the culture media. After 24 hours of culture, total RNA was extracted from the cells for the quantitative analysis of *HAS3* mRNA expression. In some experiments, supernatants were assayed for HA production. To explore the effects of IL-4, IL-13, and IFN-γ on E-cadherin expression by NHEK, NHEK were cultured with these cytokines in both low-calcium (0.15 mM Ca^{2+}) and high-calcium (1.2 mM Ca^{2+}) conditions and the expression of E-cadherin was examined 72 hours later by flow cytometry.

Organotypic culture

We used a Standard EpiDermTM kit that consists of 24 individual tissues (EPI-200), each tissue 8 mm in diameter (Kurabo), as a human skin equivalent. In the EPI-200 tissues, keratinocytes are grown on a substrate that is coated with chemically modified collagen. The EPI-200 culture inserts are grown at air–liquid interface in six-well plates. After they were equilibrated at 37 °C overnight in 900 μl of the culture medium (EPI-100-NMM; MatTek Corporation, Ashland, MA), they were then cultured using 5.0 ml of medium by placing the culture inserts atop two stainless steel washers in the six-well plates. To examine the effects of cytokines on the EPI-200 culture, we added 100 ng ml^{-1} of IL-4, IL-13, their combination, or 25 ng ml^{-1} of IFN-γ. After 4 days of the culture, EPI-200 tissues were collected and processed for hematoxylin–eosin staining, immunofluorescence staining for E-cadherin, or immunochemical staining using HABP.

Fluorescence staining of HABP and E-cadherin

Formalin-fixed paraffin-embedded tissue samples were sectioned to 4 μm and deparaffinized. Deparaffinized sections were treated with biotinylated HABP (Seikgaku Corp., Tokyo, Japan) overnight at 4 °C, followed by incubation for 1 hour at room temperature with streptavidin-Texas Red (Biosource, Camarillo, CA). For double-immunofluorescence staining, deparaffinized sections were treated with biotinylated HABP and mouse anti-human E-cadherin Ab (Dako North America Inc., Carpinteria, CA) overnight at 4 °C, followed by incubation for 1 hour at room temperature with streptavidin-Texas Red and FITC-labeled goat anti-mouse Ig (Invitrogen, Carlsbad, CA). After washing with 0.1% Tween 20/phosphate-buffered saline three

times, the slides were mounted in Perma Fluor and observed under AxioVision (Carl Zeiss, Hallbergmoos, Germany).

Quantification of mRNA expression by real-time PCR

RNA from NHEK was extracted 24 hours after stimulation using the guanidinium thiocyanate method as described by the manufacturer (ISOGEN; Nippon Gene Inc., Toyama, Japan). cDNAs were obtained from total RNA using a TaKaRa RNA PCR kit (AMV) (Takara Biochemicals, Osaka, Japan), as described by the manufacturer. Quantitative, fluorescent PCR was performed using the TaqMan system (ABI 7700; PE Applied Biosystems, Foster City, CA). We chose forward primers and reverse primers to span exon–intron boundaries. The TaqMan probes to be used with these primers were chosen with Primer Express version 1.0 (PE Applied Biosystems). The forward and reverse primers were made by Operon (Nihon Gene Research Laboratories Inc., Sendai, Japan), and the TaqMan probes were made by PE Applied Biosystems. The primers and probes used in this study are listed in Table S1 and S2. PCRs were performed in triplicate in 30 μl total reaction volume with 66 nM TaqMan probe, 400 nM forward primers, 400 nM reverse primers, and 2 × TaqMan Universal PCR Master (PE Applied Biosystems). Thermal cycling was performed for 2 minute at 50 °C to deplete contaminating RNA, with a 10-minute denaturation at 95 °C, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 minute in an ABI Prism 7700 detection system (PE Applied Biosystems). The levels of cDNA for *HAS1*, *-2*, *-3*, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) generated from cellular RNA were calculated using standard curves generated with bona-fide human cDNAs for *HAS1*, *-2*, *-3*, and GAPDH, where there was a linear relationship between the number of cycles required to exceed the threshold and the number of added copies of the cDNA (Anonymous, 1997). Triplicate data for the ratios of *HAS1*, *-2*, or *-3* mRNA/GAPDH mRNA from keratinocytes treated with various cytokines were calculated by dividing each *HAS1*, *-2*, or *-3* mRNA level by the mean of the triplicate data for GAPDH mRNA levels.

In situ hybridization

A DIG-labeled RNA probe was used to detect human *HAS3* mRNA in human skin tissues by ISH. The anti-sense RNA probe was complementary to bases 3,085–3,557 of the nucleotide sequence of the human *HAS3* gene (GenBank accession number NM_005329). For the negative control, we used a DIG-labeled mouse Mapkbp1 sense RNA probe corresponding to bases 6,258–6,648 of Mapkbp1 (GenBank accession number NM_011941). It was 491 bases in length with a CG content of 49.69%, similar to the *HAS3* anti-sense probe (Genostaff Inc., Tokyo, Japan). Formalin-fixed paraffin-embedded 5-μm sections were loaded onto a Discovery automated slide-processing system (Ventana Medical Systems Inc., Tucson, AZ). Baking and deparaffinization steps were performed as programmed in the protocol for the RiboMap ISH reagent system (Ventana Medical Systems Inc.). ISH protocols after the deparaffinization step were based on the standard protocol described in the manufacturer's RiboMap application note using a ready-to-use protease reagent (PROTEASE 2; Ventana Medical Systems Inc.). After incubation with the streptavidin–alkaline phosphatase conjugate (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 16 minutes at 37 °C, the signal was detected automatically using a BlueMap NBT/BCIP substrate kit (Ventana Medical Systems Inc.) for 3 hours at 37 °C. Finally, the sections were counterstained with a nuclear fast red-equivalent reagent, ISH Red (Ventana Medical Systems Inc) for 2 minutes before applying coverslips.

Assay of HA synthesis by keratinocytes

IFN-γ (25 ng ml^{-1}), IL-4 (10, 30, and 100 ng ml^{-1}), or IL-13 (10, 30, and 100 ng ml^{-1}) was added to the NHEK cultures and the culture supernatants were harvested 24 hours later. To measure the HA content of the culture supernatants, a sandwich type assay was performed as described previously (Rilla *et al.*, 2004). Briefly, 96-well maxisorp plates (Nunc, Roskilde, Denmark) were precoated with (nonbiotinylated) HABP (Seikgaku Corp.) to remove HA from the samples and standards (range 0–800 ng ml^{-1}). After washing, HABP labeled by a EZ-Link Maleimid Activated Horseradish Peroxidase Kit (Pierce, Rockford, IL) was added to detect the bound HA for spectrophotometric quantification using a substrate solution consisting of 0.5% 3,3′,5,5′-tetramethylbenzidine in dimethyl sulfoxide (Sigma Chemical Co., St Louis, MO) diluted 1:50 with 0.1 M sodium acetate, 1.5 mM citric acid, and 0.005% H_2O_2.

Flow cytometry

NHEK that were cultured with IL-4 (100 ng ml^{-1}), IL-13 (100 ng ml^{-1}), or IFN-γ (25 ng ml^{-1}) in low- and high-Ca conditions, were trypsinized, and further treated with 10 μg ml^{-1} of anti-E-cadherin Ab (Immunotech, Beckman Coulter Company, Marseille, France) or isotype-matched control Ab for 30 minutes followed by incubation with PE-conjugated anti-mouse Ig (Tago Immunologicals, Camarillo, CA). After washing, the expression of E-cadherin by NHEK was analyzed by FACScan using a CellQuest software (Becton Dickinson). Dead cells were gated out by adding 0.5 μg ml^{-1} propidium iodide solution.

Statistical analysis

The statistical significance of differences in the ratio of *HAS3* mRNA/GAPDH mRNA between untreated and treated NHEK in each experiment was analyzed using a paired *t*-test. To compare the data from different donors, we calculated the percent augmentation of the *HAS1*, *-2*, or *-3* mRNA/GAPDH mRNA ratio as follows:

$$\% \text{ augmentation of } HAS1, -2, \text{ or } -3 \text{ mRNA expression} = A/B \times 100$$

where A is the *HAS1*, *-2*, or *-3* mRNA/GAPDH mRNA ratio from NHEK stimulated with cytokines, and B is the *HAS1*, *-2*, or *-3* mRNA/GAPDH mRNA ratio from untreated NHEK.

The statistical significance of differences in the percent augmentation of *HAS1*, *-2*, or *-3* mRNA expression between the untreated and treated NHEK of multiple subjects was evaluated by the paired *t*-test. The statistical significance of differences in the production of HA between untreated and treated NHEK was analyzed using the unpaired *t*-test.

CONFLICT OF INTEREST

All authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Keigo Kawabata for his technical support for *in situ* hybridization. This study was supported in part by the 21st COE program of Tohoku University and the Global-COE Program from the Japanese Ministry of Education, Culture, Sports, Science and Technology; by New Energy and

Industrial Technology Development Organization; and by a grant-in-aid for scientific research from the Japan Society for the Promotion of Science (19591295 and 20790783).

SUPPLEMENTARY MATERIAL

Figure S1. *HAS1* and *Has3* mRNA expression by NHEK stimulated with various cytokines.

Table S1. Sequence of PCR primers.

Table S2. TaqMan probes.

REFERENCES

Anonymous (1997) User Bulletin #2. ABI PRISM 7700 Sequence Detection System, PE Applied Biosystems, Faster City, CA

Asherson GL, Dieli F, Sireci G, Salerno A (1996) Role of IL-4 in delayed type hypersensitivity. *Clin Exp Immunol* 103:1–4

Cher DJ, Mosmann TR (1987) Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by TH1 clones. *J Immunol* 138:3688–94

Dieli F, Asherson GL, Romano GC, Sireci G, Gervasi F, Salerno A (1994) IL-4 is essential for the systemic transfer of delayed hypersensitivity by T cell lines. Role of gamma/delta cells. *J Immunol* 152:2698–704

Fong TA, Mosmann TR (1989) The role of IFN-gamma in delayed-type hypersensitivity mediated by Th1 clones. *J Immunol* 143:2887–93

Fraser JR, Laurent TC, Laurent UB (1997) Hyaluronan: its nature, distribution, functions and turnover. *J Intern Med* 242:27–33

Fujii-Maeda S, Kajiwara K, Ikizawa K, Shinazawa M, Yu B, Koga T *et al.* (2004) Reciprocal regulation of thymus and activation-regulated chemokine/macrophage-derived chemokine production by interleukin (IL)-4/IL-13 and interferon-gamma in HaCaT keratinocytes is mediated by alternations in E-cadherin distribution. *J Invest Dermatol* 122:20–8

Grabbe S, Schwarz T (1998) Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. *Immunol Today* 19:37–44

Holden CA, Berth-Jones J (2004) Eczema, lichenification, prurigo and erythroderma. In: *Rook's Textbook of Dermatology*. (Burns T, Breathnach S, Cox N, Griffiths C, eds), 7th edn, vol. 1. Blackwell Science Ltd: Oxford, 17.11–55

Jensen PJ, Wheelock MJ (1996) The relationships among adhesion, stratification and differentiation in keratinocytes. *Cell Death Differ* 3:357–71

Laurent TC, Fraser JR (1992) Hyaluronan. *FASEB J* 6:2397–404

Maytin EV, Chung HH, Seetharaman VM (2004) Hyaluronan participates in the epidermal response to disruption of the permeability barrier *in vivo*. *Am J Pathol* 165:1331–41

Monslow J, Williams JD, Norton N, Guy CA, Price IK, Coleman SL *et al.* (2003) The human hyaluronan synthase genes: genomic structures, proximal promoters and polymorphic microsatellite markers. *Int J Biochem Cell Biol* 35:1272–83

Neis MM, Peters B, Dreuw A, Wenzel J, Bieber T, Mauch C *et al.* (2006) Enhanced expression levels of IL-31 correlate with IL-4 and IL-13 in atopic and allergic contact dermatitis. *J Allergy Clin Immunol* 118:930–7

Pasonen-Seppänen S, Karvinen S, Törrönen K, Hyttinen JM, Jokela T, Lammi MJ *et al.* (2003) EGF upregulates, whereas TGF-beta downregulates, the hyaluronan synthases Has2 and Has3 in organotypic keratinocyte cultures: correlations with epidermal proliferation and differentiation. *J Invest Dermatol* 120:1038–44

Rilla K, Pasonen-Seppänen S, Rieppo J, Tammi M, Tammi R (2004) The hyaluronan synthesis inhibitor 4-methylumbelliferone prevents keratinocyte activation and epidermal hyperproliferation induced by epidermal growth factor. *J Invest Dermatol* 123:708–14

Sakai S, Yasuda R, Sayo T, Ishikawa O, Inoue S (2000) Hyaluronan exists in the normal stratum corneum. *J Invest Dermatol* 114:1184–7

Salerno A, Dieli F, Sireci G, Bellavia A, Asherson GL (1995) Interleukin-4 is a critical cytokine in contact sensitivity. *Immunology* 84:404–9

Saunders NA, Jetten AM (1994) Control of growth regulatory and differentiation-specific genes in human epidermal keratinocytes by interferon gamma. Antagonism by retinoic acid and transforming growth factor beta 1. *J Biol Chem* 269:2016–22

Sayo T, Sugiyama Y, Takahashi Y, Ozawa N, Sakai S, Ishikawa O *et al.* (2002) Hyaluronan synthase 3 regulates hyaluronan synthesis in cultured human keratinocytes. *J Invest Dermatol* 118:43–8

Takeshita K, Yamasaki T, Akira S, Gantner F, Bacon KB (2004) Essential role of MHC II-independent CD4+ T cells, IL-4 and STAT6 in contact hypersensitivity induced by fluorescein isothiocyanate in the mouse. *Int Immunol* 16:685–95

Tammi R, Ripellino JA, Margolis RU, Tammi M (1988) Localization of epidermal hyaluronic acid using the hyaluronate binding region of cartilage proteoglycan as a specific probe. *J Invest Dermatol* 90:412–4

Trautmann A, Akdis M, Kleemann D, Altznauer F, Simon HU, Graeve T *et al.* (2000) T cell-mediated Fas-induced keratinocyte apoptosis plays a key pathogenetic role in eczematous dermatitis. *J Clin Invest* 106:25–35

Trautmann A, Altznauer F, Akdis M, Simon HU, Disch R, Bröcker EB *et al.* (2001) The differential fate of cadherins during T-cell-induced keratinocyte apoptosis leads to spongiosis in eczematous dermatitis. *J Invest Dermatol* 117:927–34

Wells AF, Lundin A, Michaëlsson G (1991) Histochemical localization of hyaluronan in psoriasis, allergic contact dermatitis and normal skin. *Acta Derm Venereol* 71:232–8

Wolff K, Kibbi A-G, Mihm MC (2003) Basic pathologic reactions of the skin. In: Freedberg IM, Eizen AZ, Wolff K, Austen KF, Goldsmith LA, Katz SI. *Dermatology in General Medicine*. 6th ed, vol. 1. McGraw-Hill: New York, 30–43

Yokozeki H, Ghoreishi M, Takagawa S, Takayama K, Satoh T, Katayama I *et al.* (2000) Signal transducer and activator of transcription 6 is essential in the induction of contact hypersensitivity. *J Exp Med* 191:995–1004