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Effects of ultraviolet B irradiation, proinflammatory cytokines and raised extracellular calcium concentration on the expression of *ATP2A2* and *ATP2C1*

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Summary

Background Darier disease (DD) and Hailey–Hailey disease (HHD) are autosomal dominantly inherited skin disorders that histologically share the characteristics of suprabasal separation and acantholysis of epidermal keratinocytes. Various mutations in the DD gene (*ATP2A2*) and the HHD gene (*ATP2C1*) (respectively encoding the calcium pumps of the sarco/endoplasmic reticulum and the Golgi apparatus) have recently been described in multiple families with DD and HHD. Mutations in *ATP2A2* or *ATP2C1* have been suggested as causing the conditions via the mechanism of haploinsufficiency. Ultraviolet (UV) B irradiation is thought to be an aggravating factor in both diseases.

Objectives To examine the effects of various stimuli on *ATP2A2* and *ATP2C1* mRNA expression, and to examine the role of calcium pumps during keratinocyte differentiation.

Methods The effects of UVB irradiation, of UVB-inducible inflammatory cytokines produced by keratinocytes and of high-calcium medium (1·8 mmol L^{-1} as opposed to 0·08 mmol L^{-1} Ca^{2+}) on *ATP2A2* and *ATP2C1* mRNA expression were quantified in cultured normal human keratinocytes using reverse transcription-polymerase chain reaction.

Results Expression of *ATP2A2* and *ATP2C1* mRNA was suppressed immediately after exposure to UVB irradiation, and modulation of mRNA expression was achieved in keratinocytes cultured with proinflammatory cytokines. The mRNA expression of both genes was increased significantly after the shift to high extracellular Ca^{2+} concentration.

Conclusions The results suggest that modulation of *ATP2A2* and *ATP2C1* mRNA expression by UV or cytokines might contribute to the clinical presentations unique to DD and HHD, and that the controlled expression of these genes plays an important role in keratinocyte homeostasis, function and differentiation.

Darier disease (DD; MIM 124200) and Hailey–Hailey disease (HHD; MIM 16960) are autosomal dominantly inherited skin disorders characterized by loss of adhesion between epidermal cells and by abnormal keratinization (acantholysis and dyskeratosis of keratinocytes). Eruptions characteristic primarily of DD, namely warty keratotic papules and plaques, appear on seborrhoeic areas such as the central trunk, scalp, forehead and flexures, and progress to blisters, erosions and mucosal lesions. The recurrent blisters and erythema in HHD develop predominantly at intertriginous areas. Ultraviolet (UV) radiation, heat and infections exacerbate these diseases.[1,2] Many patients with DD experience a flare on nonseborrhoeic areas as a delayed result of exposure to sunlight. DD lesions can be experimentally induced by exposure to UVB,[3] and UV radiation was found to provoke typical blistering in the skin of patients with HHD as well as induction of acantholytic and preacantholytic keratinocytes with condensed keratin filaments and a diminished number of desmosomes *in vivo*.[4]

DD and HHD were recently found to be caused by mutations in *ATP2A2* and *ATP2C1*, respectively, encoding the sarco/endoplasmic reticulum Ca^{2+} ATPase type 2 isoform (SERCA2) and a homologue of a yeast Ca^{2+} ATPase that pumps Ca^{2+} from the cytosol to the Golgi (PMR1).[5,6] These calcium pumps are highly expressed in human epidermal keratinocytes. Although the pathomechanism thought to underlie the autosomal dominant inheritance in these diseases is

haploinsufficiency, the mechanism directly linking the abnormality of the pumps to the characteristic pathological features is still unknown.

UVB irradiation causes skin inflammation by inducing keratinocytes to produce proinflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8 and tumour necrosis factor (TNF)-α.[7–10] In this study, we initially examined the effects of UVB irradiation on the mRNA expression levels of *ATP2A2* and *ATP2C1* in cultured normal human keratinocytes by using the on-line fluorescence quantitative reverse transcription-polymerase chain reaction (RT-PCR) system Light-Cycler®. We then determined modulations in the *ATP2A2* and *ATP2C1* mRNA levels following addition of proinflammatory cytokines to the culture.

In monolayer keratinocyte cultures, an extracellular Ca^{2+} concentration shift from low to high (0·1 mmol L^{-1} to 1·5–1·8 mmol L^{-1}) raises desmosome assembling and keratin network formation.[11,12] Because DD and HHD are disorders characterized by loss of adhesion between keratinocytes, we investigated the effect of high Ca^{2+} (1·8 mmol L^{-1}) exposure on the mRNA expression levels of both genes using real-time RT-PCR during normal keratinocyte differentiation.

Materials and methods

Cell culture

Normal human keratinocytes (derived from normal newborn foreskin) were obtained from Kurabo (Osaka, Japan) and maintained in a serum-free standard medium containing 0·08 mmol L^{-1} Ca^{2+} (Epilife-KGM®; Kurabo), supplemented with 10 μg mL^{-1} insulin, 0·1 ng mL^{-1} human epidermal growth factor, 0·5 μg mL^{-1} hydrocortisone, 50 μg mL^{-1} gentamicin, 50 ng mL^{-1} amphotericin B and 4% bovine pituitary extract, and grown at 37 °C with 5% CO_2 in 100-mm tissue culture dishes. In monolayer culture, third-passage keratinocytes were cultured until 90% confluent before exposure to UVB, cytokines or Ca^{2+} addition.

Ultraviolet B irradiation

The medium was replaced and, after washing with phosphate-buffered saline (PBS), the keratinocytes were exposed to 20 mJ cm^{-2} UVB (at this irradiation dose of UVB, the viability of cultured human keratinocytes does not decrease significantly[13,14]) from a UVB lamp (Torex FL20S/E30; Toshiba Light and Technology Co., Tokyo, Japan). After irradiation, the keratinocytes were incubated in fresh medium for 1, 3, 6 and 12 h under the same conditions.

Cytokine treatment

Further third-passage normal human keratinocytes were later placed in fresh medium to which recombinant cytokines had been added, as previously described:[15–18] 10 ng mL^{-1} IL-1α, 10 ng mL^{-1} IL-6, 10 ng mL^{-1} IL-8 or 100 ng mL^{-1} TNF-α (Genzyme, Cambridge, MA, U.S.A.). Six hours after the introduction of the cytokines, the keratinocytes were harvested and transferred to the RT-PCR.

Changing extracellular Ca^{2+} concentration

To induce differentiation, third-passage keratinocytes were switched from the standard medium containing 0·08 mmol L^{-1} Ca^{2+} to fresh culture medium containing high Ca^{2+} concentration (1·8 mmol L^{-1}) and were maintained for different periods: 1, 3, 6 and 12 h, as previously described.[19]

Quantification of *ATP2A2* and *ATP2C1* mRNA levels

Following exposure to UVB, cytokines or calcium and washing in PBS, total cellular RNA was extracted from the cultured keratinocytes using Isogen® (Wako Pure Chemical Co., Osaka, Japan), and cDNAs were synthesized by reverse transcription using an RNA PCR kit (Takara Shuzo, Otus, Japan) following the manufacturer's instructions. mRNA levels of *ATP2A2* and *ATP2C1* were analysed by RT-PCR using an on-line fluorescence PCR detection system (Light-Cycler®; Roche Diagnostics Co., Indianapolis, IN, U.S.A.). Briefly, real-time PCR was performed using the appropriate *Taq* DNA polymerase: SYBR Green I. For relative quantification, each level of mRNA was normalized to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is not modulated by UVB. The sequences of PCR primers used were as follows: *ATP2A2* mRNA exon 5, 5′-TGTATGGCAGGAAAGAAATG-3′ and 5′-TTGTCACCAACAGCAATTTCT-3′; exon 15, 5′-TATA CTTTTGTTTTGGCTTG-3′ and 5′-CAGGAAATACAGACAACTTC-3′; *ATP2C1* mRNA exon 5, 5′-CTTTGTTCAGGAATATCGTTCA-3′ and 5′-ATGACAACACCCTTTGCTTTG-3′; exon 15, 5′-AACA CAGCAGGACACACCACA-3′ and 5′-AGACGACTGGCGATTGCA ACT-3′. PCR conditions began with 2 min of denaturation at 95 °C, followed by 45 cycles of denaturation at 95 °C, annealing at 59 °C (GAPDH: 65 °C) for 10 s and extension at 72 °C for 10 s. After PCR amplification, melting curve analysis and quantification analysis were performed to confirm PCR amplification specificity. The levels of mRNA expression of both genes were compared with those of nonirradiated normal keratinocyte controls (= 1·0). All experiments were repeated five times using five different commercially available cell lines. Respective cell lines were established every week, and sent to us every week upon request. We also used one nonirradiated control cell line. Statistical analysis was done by t-test.

Results

Ultraviolet B-induced modulation of mRNA expression of *ATP2A2* and *ATP2C1*

Normal human keratinocytes were exposed to UVB radiation (20 mJ cm^{-2}) and then examined 1, 3, 6 and 12 h later for mRNA expression of *ATP2A2* and *ATP2C1*. As shown in Figure 1a, expression of *ATP2A2* mRNA was suppressed 1 and

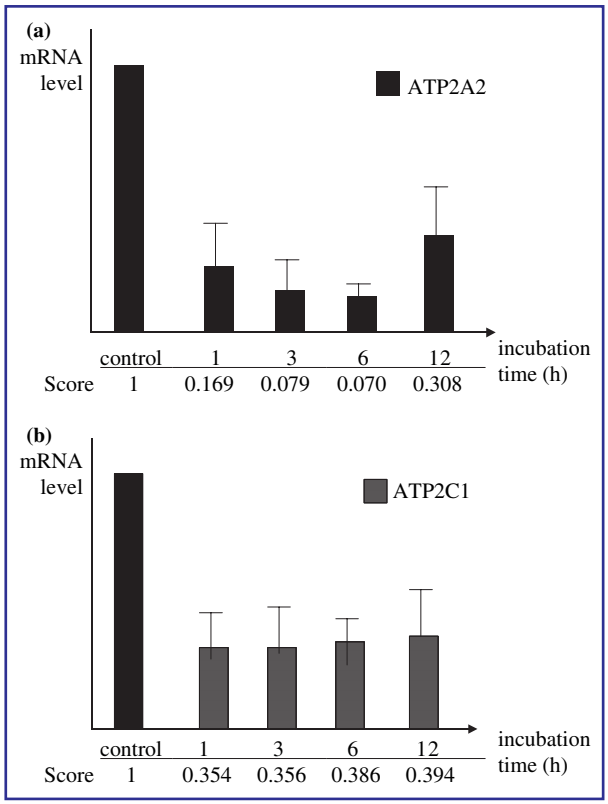

Fig 1. The effect of ultraviolet (UV) B irradiation on (a) *ATP2A2* and (b) *ATP2C1* mRNA expression. Normal human keratinocytes were exposed to UVB (20 mJ cm^{-2}). Total RNA was extracted 1, 3, 6 and 12 h after UVB irradiation. *ATP2A2* and *ATP2C1* mRNA levels were analysed by reverse transcription-polymerase chain reaction using Light-Cycler® with specific primers for the sequences of *ATP2A2* and *ATP2C1* mRNA. For relative quantification, each level of mRNA was normalized to that of glyderaldehyde-3-phosphate dehydrogenase. Results are expressed as mean ± SD.

3 h after UVB irradiation (mean ± SD relative amounts 0·169 ± 0·196 and 0·079 ± 0·105, respectively) and remained decreased 6 h after irradiation (mean ± SD relative amount 0·070 ± 0·086). Depressed mRNA expression of *ATP2A2* recovered slightly 12 h after irradiation.

Similarly, *ATP2C1* mRNA expression was downregulated by UVB as early as 1 h after irradiation. The relative mean ± SD amounts at 1, 3, 6 and 12 h after irradiation were 0·354 ± 0·069, 0·356 ± 0·141, 0·386 ± 0·054 and 0·394 ± 0·134, respectively (Fig. 1b). In contrast to *ATP2A2*, *ATP2C1* mRNA expression remained depressed up to 12 h after irradiation.

Influence of cytokines on the expression of *ATP2A2* and *ATP2C1*

Because the greatest reduction in *ATP2A2* and *ATP2C1* mRNA expression levels was observed 6 h after UVB irradiation, we incubated normal human keratinocytes with several proinflammatory cytokines for 6 h, then examined the mRNA expression levels of both genes using the Light-Cycler® system.

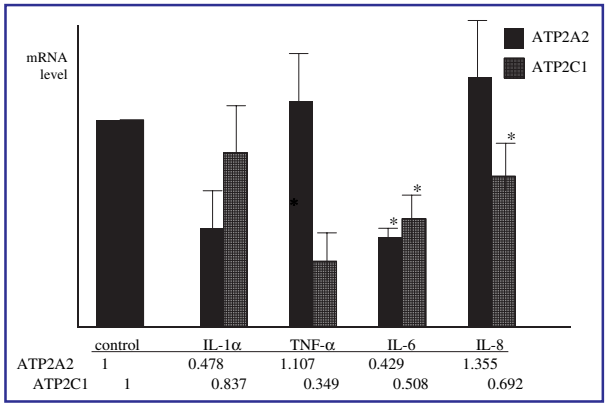

Fig 2. The effect of inflammatory cytokines on expression of *ATP2A2* and *ATP2C1* mRNA. Normal human keratinocytes were cultured with the proinflammatory cytokines interleukin (IL)-1α (IL-1a), tumour necrosis factor-α (TNF-a), IL-6 and IL-8. Total RNA was extracted from keratinocytes cultured for 6 h. *ATP2A2* and *ATP2C1* mRNA levels were analysed by reverse transcription-polymerase chain reaction. For relative quantification, each level of mRNA was normalized to that of glyderaldehyde-3-phosphate dehydrogenase. Black bar, *ATP2A2* mRNA expression level; grey bar, *ATP2C1* mRNA expression level. Results are expressed as mean ± SD. *$P < 0·05$ compared with control.

As shown in Figure 2, a significant decrease of *ATP2A2* mRNA was observed when the keratinocytes were cultured with IL-1α and IL-6 (mean ± SD relative amounts 0·478 ± 0·247 and 0·429 ± 0·093, respectively). In contrast, a slight increase in *ATP2A2* mRNA levels was observed when the cells were incubated with IL-8 and TNF-α (mean ± SD relative amounts 1·355 ± 0·688 and 1·107 ± 0·641, respectively). In contrast to *ATP2A2*, the *ATP2C1* mRNA level was downregulated in keratinocytes cultured with any of the proinflammatory cytokines used in the present experiment (mean ± SD relative amounts 0·837 ± 0·241 for IL-1α, 0·508 ± 0·133 for IL-6, 0·692 ± 0·198 for IL-8, and 0·349 ± 0·195 for TNF-α) (Fig. 2).

Effect of raised extracellular Ca^{2+} concentration on the expression of *ATP2A2* and *ATP2C1*

As shown in Figure 3, we found that expression of mRNA of both genes in cultured keratinocytes was increased during calcium-induced terminal differentiation. Normal human keratinocytes were grown to 90% confluence and then maintained in 1·8 mmol L^{-1} Ca^{2+}. Relative mRNA levels were evaluated at different times in culture. A significant increase in *ATP2A2* and *ATP2C1* mRNA was observed immediately, at 1 h after switching to high Ca^{2+} medium (mean ± SD relative amounts 1·344 ± 0·448 and 3·428 ± 1·971, respectively) and remained elevated during 24 h after exposure to raised Ca^{2+} concentration.

Discussion

The sarco/endoplasmic reticulum Ca^{2+} ATPase gene (*ATP2A2*) encodes a calcium transport pump (SERCA2), the function of

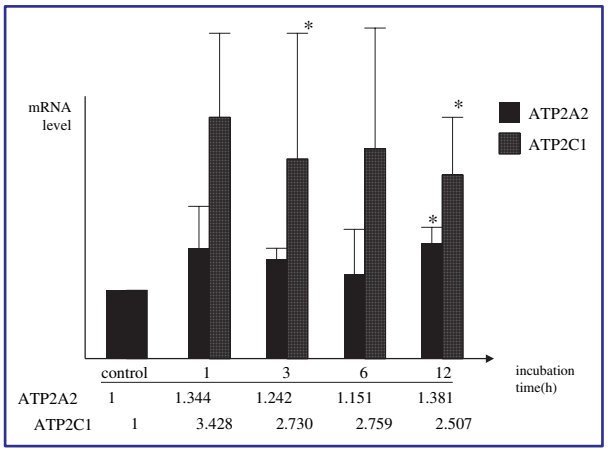

Fig 3. The effect of raised extracellular Ca^{2+} concentration on *ATP2A2* and *ATP2C1* mRNA expression. Third-passage keratinocytes cultured in standard medium containing 0·08 mmol L^{-1} Ca^{2+} were shifted to high-calcium (1·8 mmol L^{-1} Ca^{2+}) medium. Total RNA was extracted 1, 3, 6 and 12 h after changing media. Relative (normalized to glyderaldehyde-3-phosphate dehydrogenase) *ATP2A2* and *ATP2C1* mRNA levels were analysed by reverse transcription-polymerase chain reaction using Light-Cycler® with specific primers for the sequences of *ATP2A2* and *ATP2C1* mRNA. Results are expressed as mean ± SD. *$P < 0·05$ compared with control.

which is to transport calcium from the cytoplasm into the lumen of the sarco/endoplasmic reticulum (ER). The human homologue of the yeast Golgi Ca^{2+} pump is also highly expressed in human keratinocytes, where the Golgi Ca^{2+} pump accumulates Ca^{2+} and releases it in response to inositol 1,4,5-trisphosphate (IP3).[20] The Golgi apparatus shares with the ER not only the property of accumulating Ca^{2+} in the keratinocytes, but also that of rapidly releasing it upon agonist-dependent IP3 generation. Changes in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]c$) in the epidermal cells play an important role in the adhesion of one epidermal keratinocyte to another, as well as in the proliferation of these cells.

In DD and HHD, immunostaining showed a loss of the intracellular desmosomal proteins (desmoplakins 1 and 2 and plakoglobin) from desmosomes and a diffuse staining in the cytoplasm.[21] Nevertheless, HHD and normal keratinocytes did not differ in their ability to assemble desmosomes and adherens junctions.[22] No significant morphological difference between normal and HH keratinocytes was observed in culture in low (0·1 mmol L^{-1}) and high (1·1 mmol L^{-1}) Ca^{2+}-containing media. In these keratinocytes the major desmosomal proteins are biochemically intact. They are capable of assembling into desmosomes.[4]

Mutations in *ATP2A2* and *ATP2C1* are currently thought to cause DD and HHD through the mechanism of haploinsufficiency (not dominant negative nor gain of function).[5,6] Suppression of the expression from the intact allele is thought to be enough to induce skin lesions, because proteins from the mutated allele might be nonfunctional. This theory might be furthered by the discovery of the complete loss of a single allele in a kindred with HHD and by the fact that keratinocytes from this kindred showed less response to an increase in extracellular Ca^{2+} levels, elevated $[Ca^{2+}]c$ level and abnormally low $[Ca^{2+}]$Golgi level.[6] SPCA1 (protein product from *ATP2C1*) is also supposed to pump Mn in addition to Ca^{2+} however, Mn levels have not yet been evaluated in patient skin.[6,23] Futher study is required to assess the importance in changes of the Mn concentration in the pathogenesis of HHD.

On the other hand, Ca^{2+} uptake and storage by the ER were impeded in normal human keratinocytes cultured with thapsigargin (inhibitor for all SERCA family molecules).[24] Furthermore, the addition of thapsigargin or antisense oligonucleotides to SERCA2 to explant cultures of normal skin was found to induce morphological changes similar to those in DD (submitted for publication). These findings might also support the hypothesis that the pathomechanism underlying both diseases is haploinsufficiency.

Both DD and HHD symptoms are possibly aggravated by UV irradiation.[1,2] UV irradiation may trigger a cutaneous inflammatory response by directly modulating keratinocytes to upregulate specific cytokines such as IL-1, IL-6, IL-8 or TNF-α. This in turn may lead to exacerbation of DD and HHD symptoms by downregulating the expression of the responsible genes. Bearing in mind these possibilities, in the present study we examined the effects of UVB irradiation and proinflammatory cytokines on the mRNA expression levels of *ATP2A2* and *ATP2C1* in normal human keratinocytes using the on-line fluorescence quantitative RT-PCR system Light-Cycler®. As shown in Figure 1a,b, expression of *ATP2A2* and *ATP2C1* was clearly suppressed immediately after UVB irradiation and remained depressed at least up to 6 h following irradiation. Expression of *ATP2A2* was decreased by the addition of IL-1α and IL-6, but not by the addition of TNF-α and IL-8. In contrast, expression of *ATP2C1* was depressed following the addition of every type of cytokine tested, leading to the conclusion that the gene-regulating mechanism is distinct for each gene. We have recently identified the promoter region and transcription factors that can bind to the promoter region of *ATP2C1* (manuscript in preparation). Differences in the sequences of the promoter regions in the respective genes might account for the gene-regulating mechanism distinct for each gene. It is also well known that UV irradiation can induce production of several other molecules such as prostaglandin E, transforming growth factor-β, vascular endothelial growth factor, basic fibroblast growth factor, nerve growth factor and neuropeptides.[25] Further study evaluating the effect of these molecules on the expression of calcium pumps is necessary to assess the involvement of these molecules in the pathogenesis of DD or HHD.

The function of the two calcium pumps is thought to fall far short of normal activity in diseased keratinocytes, due to the defective allele inhibited by gene mutations and the suppression of the intact allele by UVB and/or inflammatory cytokines. Extreme reduction in the total activity of the calcium pumps over the threshold could cause continued low levels of $[Ca^{2+}]$ER or $[Ca^{2+}]$Golgi, thereby maintaining increased $[Ca^{2+}]c$ in the keratinocytes. These observations

suggest that modulation of *ATP2A2* and *ATP2C1* mRNA expression by UV or cytokines might contribute to the formation of the lesions seen in DD and HHD, and that different responses seen in the expression of each gene to several different stimuli might contribute to the formation of unique (typical) clinical presentations of DD and HHD, respectively. Notably, changing the extracellular Ca^{2+} concentration itself can also modulate the expression of different types of calcium pump and influences keratinocyte homeostasis in a complex manner, as observed in this experiment. It may be possible that some agents that raise the mRNA level of the intact calcium pump allele might also help to maintain normal $[Ca^{2+}]$ER, $[Ca^{2+}]$Golgi and $[Ca^{2+}]$c in keratinocytes, consequently normalizing cell–cell contact and dyskeratosis in DD and HHD. Finally, the relevance of these results to SERCA2 and SPCA1 protein levels remains uncertain. We will examine the effects of various stimuli on the expression of these proteins in future work.

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