Previous investigations have indicated that the generalised rash caused by TCE is mediated by a delayed-type hypersensitivity mechanism [2, 11, 18, 19]. As shown in table 3, 2 cases showed positive results for TCE patch testing [18, 19], whilst the case reported by Phoon et al. [20] was negative for TCE. Nakayama et al. [11] demonstrated a positive reaction for TCE and its metabolite trichloroethanol, but not for trichloroacetic acid. Our patch test was positive for trichloroethanol, trichloroacetic acid and chloral hydrate, but not for TCE. Previously, we suggested that a metabolite of trichloroethylene, chloral hydrate, which has been reported to be a causative agent of severe rash and itching, might be another candidate responsible for the generalised skin disorders due to TCE exposure [1]. The positive results for chloral hydrate on patch testing and the drug-induced lymphocyte stimulation test strongly support this suggestion. Taken together, oxidative metabolites of TCE, which might include trichloroacetylated protein adducts, could induce the generalised skin eruption.

DIHS [4–7], which is also referred to as DRESS [8, 9], is characterised by a serious adverse systemic reaction that usually appears after 3–6 weeks of exposure to certain drugs such as anticonvulsants and allopurinol [4–7]. DIHS is frequently associated with the reactivation of HHV-6 [4–7, 9], and the reactivation of HHV-7, CMV or Epstein-Barr virus has also been reported [7, 21, 22]. When both HHV-6 and CMV are reactivated in the same patients with DIHS, HHV-6 DNA is detected 21–35 days after onset, followed 10–21 days later by positivity for CMV DNA; the CMV IgG antibody titre is also elevated 10–21 days after elevation of the HHV-6 antibody titre [23]. In our patient, serum HHV-6 and CMV DNA were detected 4 and 7 weeks, respectively, after onset; the IgG antibody titres to HHV-6 and CMV were significantly elevated 6 and 9 weeks, respectively, after the onset. Therefore, the period of exposure before disease onset and the period of reactivation of these two viruses after onset were concordant with those in DIHS. Hypogammaglobulinaemia is also a characteristic finding in DIHS [24]. The immunoglobulin levels in our patient at the early stage were within the normal range, whilst these levels were relatively low compared with those after resolution, as shown in table 1.

The cutaneous manifestations of TCE exposure are classified into two categories: HS and erythema multiforme/Stevens-Johnson syndrome/toxic epidermal necrolysis [1]. Based on this categorisation, 52% of cases are classified as the former and 48% as the latter [1]. Our patient did not have severe mucous membrane involvement or any evidence of epidermal necrosis like those in Stevens-Johnson syndrome or toxic epidermal necrolysis in biopsy specimens. The patient's symptoms fulfilled all of the following diagnostic criteria for DIHS established by the Japanese Research Committee on Severe Cutaneous Adverse Reactions, as reported previously [25]: maculopapular rash developing 3 weeks after starting with an agent; prolonged clinical symptoms 2 weeks after discontinuing the causative agent; fever of >38 °C; liver abnormalities; leucocyte abnormalities such as leucocytosis ($>11 \cdot 10^9 \cdot l^{-1}$), atypical lymphocytosis (>5%) and eosinophilia ($>1.5 \cdot 10^9 \cdot l^{-1}$); lymphadenopathy, and HHV-6 reactivation. Other cases reported as TCE-related HS [11, 15–19, 26–28] also met most of these criteria for DIHS. Several findings that are useful for diagnosing TCE-induced skin lesions are shown in table 4.

Recently, HLA-B*1301 was identified as a marker of individual susceptibility for TCE-induced HS with an odds ratio of 27.5 in a patient group in China [13]. HLA-B*1301 was present in our Japanese patient. This finding is analogous to carbamazepine-induced Stevens-Johnson syndrome and allopurinol-induced severe cutaneous adverse reactions, which have strong associations with HLA-B*1502 [31] and HLA-B*5801 [32], respectively. In China, the allele frequency of HLA-B*1301 in the southern population is higher than that in the northern population, and is considered one of the possible major factors in the mass outbreaks of TCE-induced hypersensitivity dermatitis in the southern population [13]. The allele frequency in Japan is 1.3% [33], which is even lower than that (3.64% [34]) in the Han population in northern China, and may partly explain the reason for the fewer cases in Japan than in China. As another genetic susceptibility factor, the polymorphism of ALDH, the major enzyme in TCE metabolism, was reported to be associated with TCE-induced HS [14]. Li et al. [14] demonstrated that the frequency of heterozygous ALDH2 *1/*2 plus homozygous ALDH2 *2/*2 in patients with this disease was significantly lower than in exposed controls. The ALDH polymorphism in our patient was homozygous ALDH2 *1/*1. Further cases to identify genetic susceptibility markers associated with TCE-induced HS should be accumulated to explore the roles of tumour necrosis factor-α [35] and N-acetyltransferases [36].

The reported mortality amongst patients with TCE-related HS is 9% [2]. Our case serves as a reminder that workers exposed to TCE may develop severe generalised skin reactions that resemble DIHS/DRESS.

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Protein kinase C δ and η differently regulate the expression of loricrin and Jun family proteins in human keratinocytes

Nagisa Kamioka [a,b,f], Tomoko Akahane [c], Yoko Kohno [d], Toshio Kuroki [e], Masafumi Iijima [b], Ikuo Honma [f], Motoi Ohba [a,*]

[a] Institute of Molecular Oncology, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan
[b] Department of Dermatology, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan
[c] Department of Obstetrics and Gynecology, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo, Japan
[d] Department of Oral Pathology, Showa University School of Dentistry, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan
[e] Research Center Science Systems, Japan Society for the Promotion of Science, 6 Ichibancho, Chiyoda-ku, Tokyo, Japan
[f] Department of Physiology, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan

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ABSTRACT

Barrier function of the epidermis is maintained by precise expression of keratinocyte-specific structural proteins to form the cornified cell envelope (CE). Loricrin, a major component of the CE, is expressed at the late stage of keratinocyte differentiation. In this study, we reveal the isoform-specific function of protein kinase C (PKC) in the regulation of loricrin expression. Both PKCδ and PKCη have been recognized as differentiation-promoting isoforms. However, loricrin expression was inversely controlled by PKCδ and PKCη in cultured keratinocytes and 3D skin culture; i.e. loricrin expression was decreased by PKCδ and increased by PKCη. To clarify the mechanisms that PKCδ and PKCη oppositely regulate the loricrin expression, we examined the expression of activator protein-1 (AP-1) family proteins, which modulate the transcription of loricrin and are downstream molecules of PKC. PKCδ decreased c-Jun expression, whereas PKCη increased JunD, which are positive regulators of loricrin transcription. These findings suggest that inverse effects of PKCδ and PKCη on loricrin expression attributes to the expression of c-Jun and JunD.

1. Introduction

The epidermis is the major component to defend the organism against irritants in its external surroundings. The multilayer structure of the epidermis is maintained by a finely tuned balance of growth, differentiation and cell death. Once the basal cells detach from the basement membrane, they proceed to terminal differentiation. They exit the cell cycle and sequentially induce a set of differentiation-associated genes including keratin1/10 (K1/K10), SPRs, involucrin and loricrin [1,2]. Eventually, activation of transglutaminase 1 (TGase 1) leads to the formation of the cornified envelope by cross-linking its substrate proteins such as loricrin.

Protein kinase C (PKC) is a serine/threonine kinase which regulates a wide variety of cellular functions including keratinocyte differentiation [3–5]. PKC consists of 10 isoforms which are classified into conventional (α, β, γ), novel (δ, ε, η, θ, μ) and atypical (ζ, λ/ι) subfamilies based upon their molecular structure and co-factor requirements [6]. Among them, five PKC isoforms are expressed in keratinocytes [7], i.e. PKCα, δ, ε, η and ζ. In particular, PKCη has a unique feature in the skin; it is specifically expressed in the differentiated epithelial tissues including the upper layer of epidermis. PKCη is associated with Fyn tyrosine kinase in keratinocytes, leading to the inactivation of CDK2 [8]. Moreover, in our previous study, PKCη induces the cell cycle arrest at G1 phase and the terminal differentiation by the activation of TGase 1 [4].

Some studies have shown that PKCη shares the common features with PKCδ. PKCδ also inhibits the cell proliferation and activates TGase1 [4]. Involucrin mRNA and proteins are increased by PKCδ and PKCη via the MEKK1/MEK3/p38δ MAPK pathway [9,10]. Schindler et al. reported that PKCδ and PKCη elicit proline-rich protein tyrosine kinase (Pyk2) activation, resulting in the increase of promoter activity and protein level of involucrin [11]. However, PKCδ and PKCη possess discrete properties in the activation and down-regulation, subcellular localization and its associated proteins [12–15]. PKCδ is located in whole layers of the epidermis, while PKCη is restricted to the differentiated layer [16]. PKCδ promoted the caspase-3 activity during UV-induced apoptosis, whereas PKCη reduced its activity [17]. These findings suggest the possibility that PKCδ and PKCη have specific function in keratinocytes.

* Corresponding author. Fax: +81 3 3784 2299.
E-mail address: moba@pharm.showa-u.ac.jp (M. Ohba).

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In the current study, we evaluated the roles of PKCδ and PKCη in keratinocyte differentiation by suspension culture. Furthermore, we used a three-dimensional (3D) skin culture to examine the functions of PKCδ and PKCη in the epidermis. We describe here that PKCδ and PKCη have opposite abilities to modulate the expression of loricrin and diversely affect the expression of Jun family transcriptional factors, which are known as downstream molecules of PKCs.

2. Materials and methods

2.1. Antibodies

Anti-human keratin 1, keratin14, anti-involucrin, and loricrin antibodies were from Covance (Richmond, CA). Anti-PKCδ (C-20), c-fos (H-125), c-Jun (H-79), JunB (C-11) and JunD (329) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-PKCη antibody (GTX24134) was from GeneTex Inc.

2.2. Cell culture and 3D skin culture

Primary normal human keratinocytes (NHK) were purchased from Lonza (Walkersville, MD) and cultured in serum-free keratinocyte growth medium2 (KGM2, Lonza) including human keratinocyte growth supplement as described previously [4] For suspension-induced differentiation, NHK were detached with 0.025% trypsin and 0.02% EDTA and suspended in 1.45% methylcellulose-containing KGM2 on polyhydroxyethylmethacrylate (poly-HEMA)-coated dishes [18]. *In vitro* skin reconstitution (3D culture) was prepared as described previously [19]. Briefly, the dermal equivalent was made with 2.5×10^5 normal human dermal fibroblasts (Lonza) per collagen (Collagen Type A, 2.4 mg/ml, Nitta Gratin, Osaka, Japan) in cell culture inserts (1.0 mm pore size, PET track-etched membrane, Becton Dickinson Lab ware, Franklin lakes, NJ). NHK were seeded on the collagen matrix at the density of 1.2×10^6 cells per gel. The medium was replaced with KGM2: FAD = 1:1 after 24 h of seeding and the culture was further continued for 24 h. The gel surface was raised to the air–liquid interface. After 9 days, the gel was embedded in Tissue-Tek OTC compound (Sakura Finetek) and placed into liquid nitrogen for frozen section or fixed with formalin for paraffin-embedded section.

2.3. Adenovirus-mediated gene transfer

Replicative-deficient Ad5 type adenovirus vectors of wild and the kinase negative mutants of mouse PKCδ and mouse PKCη were constructed as described previously [4]. Kinase negative mutants of PKC were generated by substitution of arginine or alanine for lysine at the ATP binding site of PKC [17]. Purification and titration of adenovirus was performed with Adeno-X adenovirus purification kit and Adeno-X rapid titer kit (Clontech) according to the manufacturer's instructions. For introducing the adenovirus vectors into 3D cultures, adenovirus (1.0×10^8 pfu/gel) was added both onto the keratinocytes and into the medium of the culture 1 day before air-exposure of the gel.

2.4. Real-time PCR

Total RNA was extracted from the cells by using Illustra RNA spin kit (GE Healthcare) according to the manufacture's protocol. One microgram of total RNA was reverse-transcribed to cDNA by using Superscript III reverse transcriptase (Invitorgen Inc.) and random primers (Promega). Gene expression was measured by quantitative RT-PCR by using ABI PRISM 7000 or StepOne Real-Time PCR System (Applied Biosystems) and SYBR Green method (SYBR GreenER qPCR SuperMix, Invitrogen). Expression levels of mRNA were normalized to corresponding glyceraldehyde-3-phosphate de-hydrogenase (GAPDH) house keeping gene. The sequence of the primer pairs, 5′ and 3′, were as fellows: K1, AGGGGGCTTC AGCTCTGGCT and TGGTGGTCCTGCGCTGGTAGT; involucrin, TCCAC TGCCTCCCCCATGCC and GCAGCTCCTGCTCCTGTGGC; loricrin, GG AGTTGGAGGTGTTTTCCA and CAAACCTCGGGTAGCATCAT; SPR2a, TGGTACCTGAGCACTGATCTGCC and CCAAATATCCTTATCCTTTCTT GG; c-Jun, GTACCTGATGAACCTGATGC and GGTCACAGCACATGCCA CTT; JunB, GTCACCGAGGAGCAGGAGG and TCTTGTGCAGATCGTCCA GG; JunD, TGACGCTGAGCCTGA GTGAG and TCGGGAGAGGCGAGCA.

2.5. RNA interference

For transient RNA interference, PKCδ siRNA (sc-36353) from SantaCruz Biotech was transfected into NHK with Lipofectamine RNAiMAX (invitrogen). Twenty-four hours after transfection, the cells were trypsinized from the dishes and cultured in the suspension medium as described above. Control siRNA-A (sc-37007, SantaCruz Biotech.), which consists of a scrambled sequence, was used as a negative control.

2.6. Immunoblotting

Whole cell lysates were prepared by lysing the cells in 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 2.5 mM sodium pyrophosphate, 20 mM NaF, 50 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF. Lysates were centrifuged for 10 min at 4 °C and an equal amount of protein from the supernatants was used for SDS–PAGE and immunoblotting.

2.7. Immunohistochemistry

3D cultures were fixed with 3.7% formaldehyde and embedded in paraffin. Sections were de-waxed and boiled in 10 mM citrate buffer (pH 6.0) in a microwave oven at 500 W for 10 min. For staining of Jun/Fos family genes, the specimen were blocked with 2% goat serum and incubated with the primary antibody. Proteins were detected with Histofine Max-PO kit (Nichirei, Tokyo). Frozen cryostat sections were fixed with ethanol: acetone = 1:1 at −20 °C for 10 min. After washing with phosphate-buffered saline (PBS), the specimen was blocked with 2% goat serum and incubated with the appropriate primary antibodies against the keratinocyte differentiation markers or PKC isoforms. After additional three washes with PBS, the sections were visualized with Alexa488- or Alexa546-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (Invitrogen). Nuclear staining was performed with DAPI.

3. Results

3.1. Opposite effects of PKCδ and PKCη on loricrin induction during suspension-induced differentiation of keratinocytes

Throughout the present study, we used the adenovirus vectors expressing dominant negative mutants of PKCδ (D/Nδ) and PKCη (D/Nη) to inhibit each kinase activity in keratinocytes. First, we examined the expression of various differentiation markers in suspension-induced differentiation of NHK introduced D/Nδ and D/Nη. Real-time PCR analysis revealed that K1 mRNA expression, an early marker of differentiation, was significantly increased by D/Nδ and D/Nη. Induction of SPR2a and involucrin genes, upper spinous and granular layer markers, were decreased in D/Nδ- and D/Nη-expressing keratinocytes (Fig. 1A). These results suggest that both PKC isoforms function as positive regulators in the mid/late stage

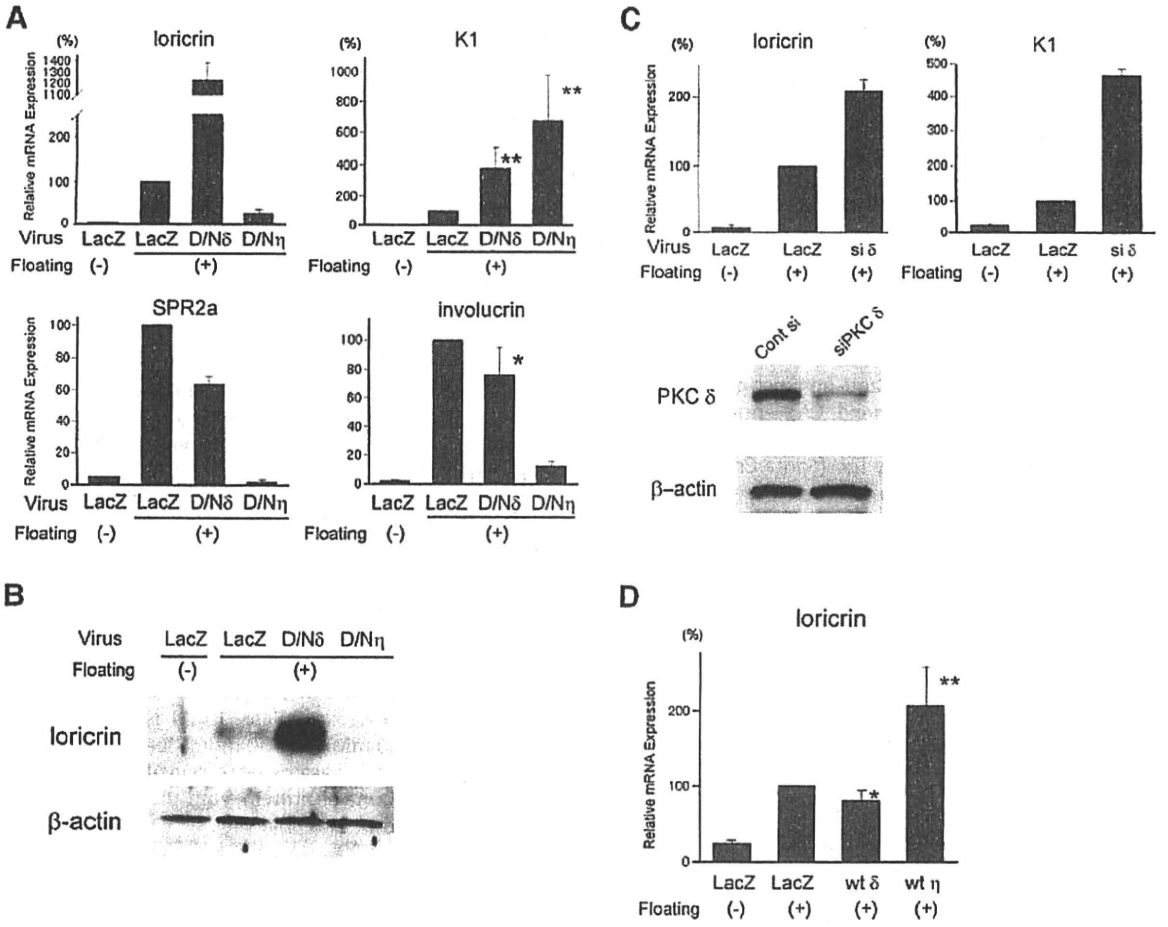

Fig. 1. PKCδ and PKCη exhibit opposite effects on the expression of loricrin in human keratinocytes. (A) Real-time PCR analysis of the differentiation marker in human keratinocytes differentiated by suspension culture (Floating). Twelve hours after infection with the adenovirus vector of β-galactosidase (LacZ), or the dominant negative PKCδ (D/Nδ) or PKCη (D/N η), normal human keratinocytes (NHK) were induced to differentiation by the suspension for 48 h. Values represent means ± standard deviations of triplicate determinations and are shown as a relative ratio to the value for the suspended lacZ-infected cells. (B) Western blot analysis of loricrin in dominant negative PKC-expressing NHK. Differentiation was induced by suspension for 60 h. (C) Effects of PKCδ siRNA on the induction of loricrin and K1 mRNA. (D) Effects of wild type PKCδ and PKCη on the induction of loricrin by the suspension culture. Cells were subjected to the suspension culture for 36 h. MOI of the adenovirus vectors were at 15 in all experiments. Asterisks indicate the significant difference from the suspended LacZ-infected cells ($P < 0.1$, $^{**}P < 0.05$).

of keratinocyte differentiation. However, only the loricrin expression, a late marker of differentiation, was inversely affected by D/Nδ and D/Nη (Fig. 1A, upper left). D/Nη inhibited the induction of loricrin significantly, whereas D/Nδ increased strikingly. These contrastive effects between PKCδ and PKCη were verified by the experiment of immunoblot shown in Fig. 1B. The above results were unexpected, because 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) increases loricrin in keratinocytes [3]. Therefore, we confirmed the effects of PKCδ on suspension-induced differentiation by using PKCδ siRNA. Compared to scramble siRNA-transfected NHK, the loricrin mRNA level was elevated in the siPKCδ-transfected cells (Fig. 1C). K1 expression was also increased by PKCδ siRNA. Furthermore, we examined the effects of wild type PKCδ (wtδ) and PKCη (wtη). The gain of function analysis revealed the inverse effects on the loricrin expression to the loss of function analysis shown above (Fig. 1A–C). Overexpression of wtδ slightly reduced the expression of loricrin, while wtη increased it (Fig. 1D). These results indicate that PKCδ represses the loricrin expression and PKCη increases it during the suspension-induced differentiation.

3.2. Inhibition of PKCδ increases the loricrin expression in 3D culture

To elucidate the role of PKCδ and PKCη in the epidermal differentiation in more physiological condition, we explored the influences of the dominant negative PKCδ or PKCη in *in vitro* reconstituted skin equivalent (3D culture). The D/Nδ or D/Nη adenovirus vector was transduced into 3D culture before forming the cornified layer and lifting to the air-exposure. Elevated expression of either D/Nδ or D/Nη was confirmed in the 3D culture even after 9 days of infection (Fig. 2A). HE staining showed the moderately thicker epidermis in both D/Nδ and D/Nη-introduced 3D cultures than LacZ-transduced control culture (Fig 2B, top panel). In accord with the results seen in Fig. 1, loricrin expression was inversely affected by PKCδ and PKCη (Fig. 2B). In the D/Nδ-introduced 3D culture, the expression of loricrin apparently increased in the granular layer, and its weak expression can be seen even in some cells of the spinous layer, suggesting that PKCδ suppresses the expression of loricrin in the epidermis. In contrast, loricrin expression decreased in the D/Nη-introduced 3D culture (Fig. 2). Expression of involucrin was attenuated by both D/Nδ and D/Nη, and K1 was increased (Fig. 2B).

3.3. Inhibition of PKCδ and PKCη results in the different expression pattern of AP-1 family genes

Loricrin gene harbors the AP-1 binding sites in the promoter, and Jun family proteins mainly regulate the transcriptional activity of loricrin [20]; c-Jun and JunD increase the transcription activity,

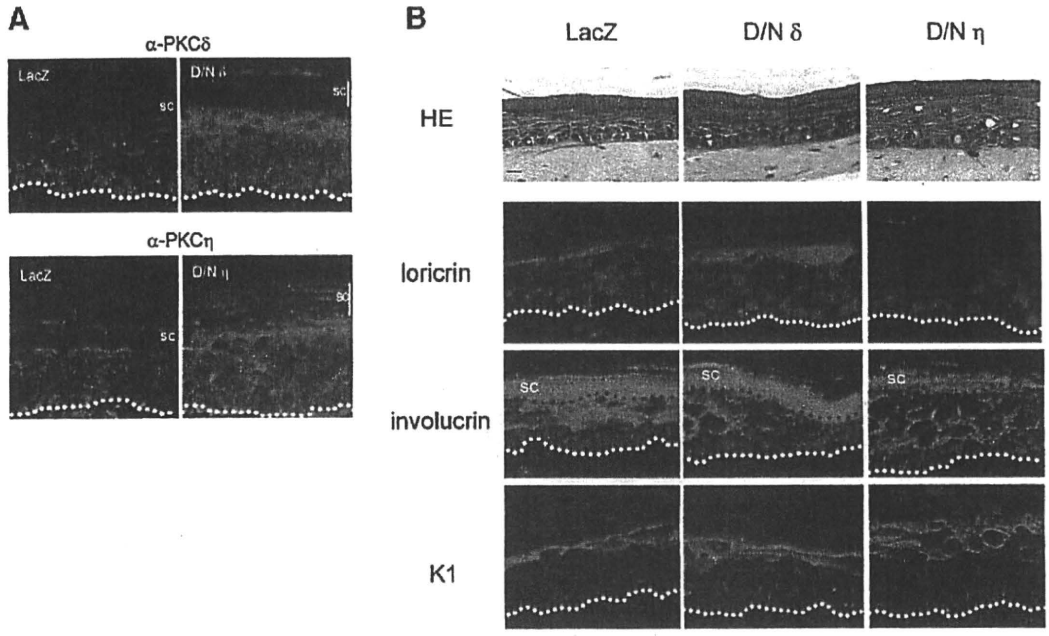

Fig. 2. Expression of differentiation marker proteins in 3D skin culture introduced the dominant negative PKC adenoviruses. (A) Expression of PKC isoforms in the adenovirus-infected 3D skin culture. Keratinocytes in the 3D culture were infected with adenovirus vectors of lacZ, D/Nδ or D/Nη as described in the Material and methods. Expression of each PKC (upper panel: PKCδ, lower panel: PKCη) was detected at 9 days after air-exposure. (B) Sections from the 3D cultures were stained with hematoxilin–eosin (HE) or immunostained for the differentiation markers:loricrin, involucrin and K1. Nuclei were stained with DAPI. The white dots and the black dots represent the boundaries between the epidermis and the dermis, the granular and cornified layers, respectively. sc: stratified cornium, Scale bar; 20 μm.

while JunB decreases it. Furthermore, AP-1 transcription factors are known to be a nuclear target of the PKC signaling pathway [21]. Therefore, we examined the expression of AP-1 proteins in D/Nδ or D/Nη-introduced 3D culture to clarify the mechanisms that both PKC isoforms play opposite roles in the loricrin expression. Immunohistochemical analysis showed the apparent different expression pattern of Jun/Fos families between D/Nδ or D/Nη-introduced 3D culture. D/Nδ increased c-Jun and JunD expression, especially in the differentiating layer (Fig. 3A). Little alteration of JunB in D/Nδ-introduced 3D culture was observed. In contrast, D/Nη significantly decreased JunD expression and moderately JunB. In addition, c-Jun was localized in both the cytoplasm and nuclei in D/Nη-expressing 3D culture, while it was present in the nuclei in LacZ- and D/Nδ-expressing 3D culture (Fig. 3A). Slight increase of c-Fos expression was observed in both D/Nδ- and D/Nη-expressing 3D culture (Fig. 3A). Finally, we examined the mRNA expression of Jun family genes in suspension culture of keratinocytes. In a similar way, D/Nδ significantly increased c-jun and modestly JunD, while D/Nη decreased them (Fig. 3B). These results suggest that PKCδ down-regulates the loricrin expression by the decrease of c-Jun, while PKCη up-regulates it by the increase of JunB and JunD in the epidermis.

4. Discussion

The structure and function of the epidermis are maintained by precise execution of the complex differentiation program, including the induction of the cornified envelope precursor proteins and the activation of a variety of protein kinases. PKC fulfills critical roles in the differentiation process of keratinocytes [5,8]. In the present study, we propose a novel function of PKCδ and PKCη in the expression of loricrin and AP-1 transcription factors in keratinocytes.

We showed that the inhibition of PKCδ increased loricrin expression, indicating the negative function of PKCδ in the late stage of differentiation (Figs. 1 and 2). However, several lines of evidence have shown that PKCδ is a differentiation inducer. The expression level of PKCδ is apparently elevated in differentiating keratinocytes [7]. Overexpression of PKCδ gives rise to the activation of TGase1 and the induction of desmoglein 1, an adhesion molecule in the differentiated keratinocytes [4,22] In current study, we also display that PKCδ is involved in the induction of involucrin and SPR2a genes (Figs. 1 and 2). Therefore, PKCδ acts as a negative regulator of differentiation only for the loricrin expression. Our data regarding loricrin is the first evidence to show the inhibitory effect of PKCδ in keratinocyte differentiation.

In contrast, the suppression of PKCη prevented loricrin expression as well as involucrin and SPR2a. Considering together the previous reports [4,8,23] and present results, PKCη appear to possess only the ability to induce the terminal differentiation of keratinocytes. PKCη is mainly expressed in the granular layers [16], while PKCδ is localized throughout the epidermis. These findings suggest that the limited distribution of loricrin in the granular layer results from the balance between the positive effect by PKCη and the negative effect by PKCδ on loricrin expression.

In some previous works, the gene function was analyzed in 3D culture by using lentivirus vectors or stable transformants of HacaT cells, an immortalized human keratinocytes [5,24]. In this study, we utilized the combination of 3D skin culture with adenovirus-mediated gene transfer to elucidate PKC function. The adenovirus vector is more useful to introduce desired genes into 3D culture. It is not necessary to establish the stable transformants and is possible to introduce one or more genes at the same time only by adding the adenoviruses to 3D culture. Dose-dependent effects of introduced gene can be easily examined by increase of the concentration of adenovirus.

The experiments using 3D cultures revealed that PKCη positively regulated JunB and JunD expression, whereas PKCδ negatively modulated c-Jun expression. JunB suppresses proliferation of keratinocytes and promotes stratification [24]. JunD and JunB, but not c-Jun, increase the involucrin transcriptional activity [25,26]. Moreover, JunD activates the transcription

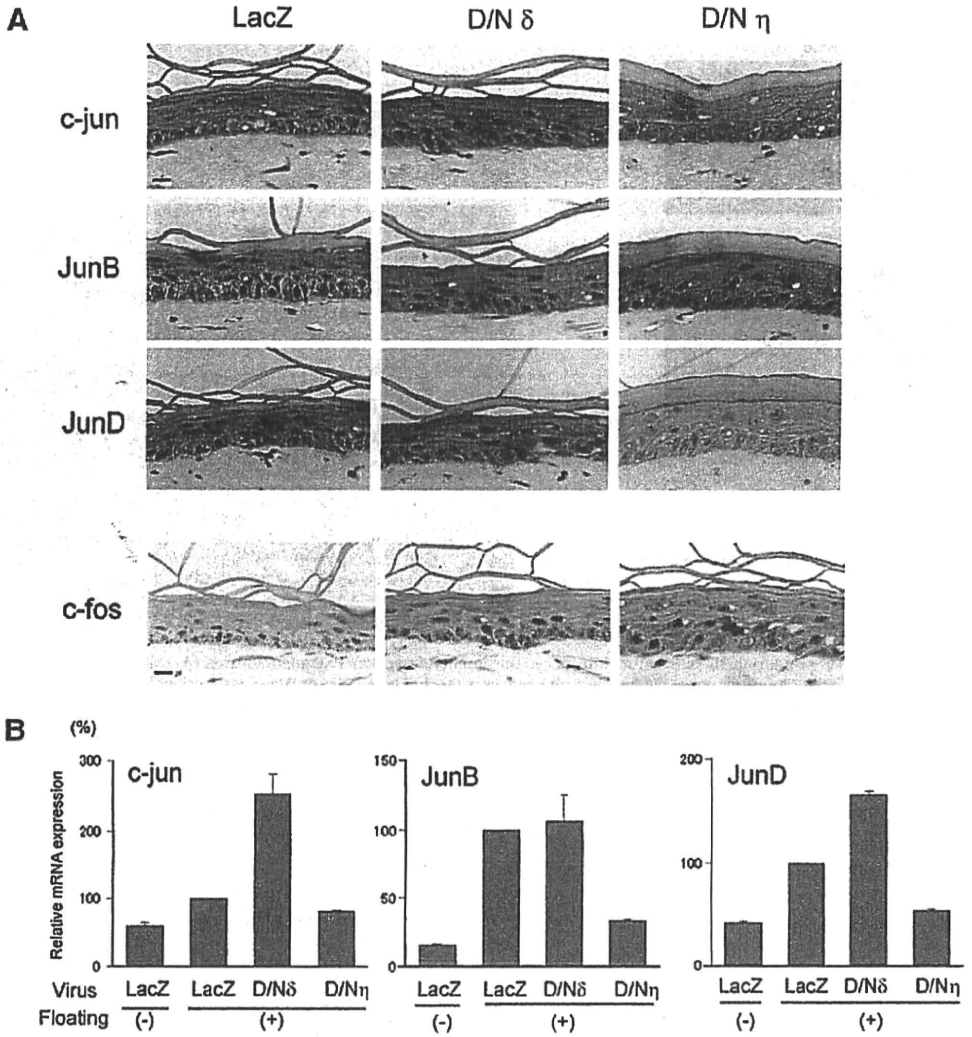

Fig. 3. (A) Dominant negative PKCη decreases the expression of JunB and JunD proteins in 3D skin culture. (A) LacZ, D/Nδ or D/Nη adenovirus vector was infected into 3D culture for 9 days. The sections were immunoreacted with c-Jun, JunB, JunD and c-fos specific antibodies and visualized with AEC dye. The sections were counterstained with hematoxilin. Scale bar; 20 μm. (B) Effects of dominant negative PKC on the mRNA expression of Jun family genes in NHK induced to differentiation by suspension culture. Values represent means ± standard deviations of triplicate determinations and are shown as a relative ratio to the value for the suspended lacZ-infected cells.

activity of fillaggrin, a late marker of differentiation [27]. On the other hand, c-Jun enhances proliferation and tumor formation of the skin [28]. c-Jun-deficient keratinocytes poorly proliferate and show increased differentiation, accompanied by decreased expression of EGFR [29]. These findings suggest that keratinocyte differentiation (except for the loricrin expression) is promoted by PKCη through the up-regulation of JunB/JunD and by PKCδ through the down-regulation of c-Jun. The distinct regulation of loricin expression from other differentiation-associated genes by PKCδ might attribute to the dependence on c-Jun for its transcriptional activation [20,30]. The expression of loricrin is also controlled by some other factors such as Sp-1, AP-2 and p300/CBP [30]. Further exploration is needed to clarify the precise regulation mechanisms of the expression differentiation marker by PKC isoforms.

In conclusion, we present the evidence that PKCδ and PKCη inversely regulate the loricrin expression, although both isoforms similarly control the expression of other differentiation markers. Moreover, the expression of AP-1 family genes is also diversely modulated by PKCδ and PKCη. These findings help to understand the complicated signaling pathway of human keratinocyte differentiation.

Acknowledgments

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ORIGINAL PAPER

Involvement of IL-17F via the induction of IL-6 in psoriasis

Sawa Fujishima · Hideaki Watanabe · Mio Kawaguchi · Takao Suzuki · Satoshi Matsukura · Tetsuya Homma · Brandon G. Howell · Nobuyuki Hizawa · Toshiyuki Mitsuya · Shau-Ku Huang · Masafumi Iijima

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Abstract Recently, the important role of T helper 17 (Th17) cells in psoriasis has been clarified; however, the role of IL-17F produced by Th17 cells is still not fully understood. IL-6 exhibits multiple biologic functions, such as regulation of immunological responses including those in psoriatic reactions. Therefore, we examined the production of IL-6 protein in normal human epidermal keratinocytes (NHEKs) stimulated by IL-17F, TNF-α, IL-17A, and IL-17A in combination with TNF-α, and PBS control. We then examined the expression of *IL-6* mRNA in mouse skin after intradermal injection of IL-17F. Finally, IL-17F expression in skin biopsy specimens from psoriasis patients was examined by immunohistochemistry. The results showed that IL-17F induced production of IL-6 in NHEKs in a time-dependent manner. This could be attenuated by chimeric inhibitor blocking the IL-17 receptor. The amounts of IL-6 stimulated by IL-17F were much higher than those stimulated by TNF-α or IL-17A. IL-6 was also significantly upregulated via synergistic stimulation with IL-17A plus TNF-α. The expression of *IL-6* mRNA 24 h after IL-17F injection in the mouse skin was 3.2-fold higher than that in the control group. Immunohistochemistry of inflammatory cells in the dermis demonstrated a large number of $CD4^+$ T cells showing IL-17F positivity in psoriatic skin lesions, but few or none in non-lesional psoriatic skin. Our results indicate that IL-17F produced by $CD4^+$ T cells causes the inflammation in psoriasis partly through induction of IL-6 in keratinocytes.

Keywords IL-17F · Th17 · IL-6 · Psoriasis · Keratinocyte

S. Fujishima · H. Watanabe (✉) · M. Iijima
Department of Dermatology,
Showa University School of Medicine, 1-5-8 Hatanodai,
Shinagawa-ku, Tokyo 142-8666, Japan
e-mail: hwatanabe@med.showa-u.ac.jp

M. Kawaguchi · N. Hizawa
Department of Respiratory Medicine,
Institute of Clinical Medicine, University of Tsukuba,
Ibaraki, Japan

T. Suzuki · T. Mitsuya
Department of Pathology,
Showa University Fujigaoka Hospital, Yokohama, Japan

S. Matsukura · T. Homma
Department of Respiratory and Allergy Medicine,
Showa University School of Medicine, Tokyo, Japan

B. G. Howell
Division of Dermatology, University of Toronto,
Toronto, ON, Canada

S.-K. Huang
Asthma and Allergy Center, Johns Hopkins University,
Baltimore, MD, USA

Abbreviations

NHEKs	Normal human epidermal keratinocytes
IL	Interleukin
Th	T helper

Introduction

Psoriasis is generally believed to be a complex autoimmune inflammatory disease with a genetic basis [2, 30]. IL-6 is a multifunctional inflammatory cytokine produced by T cells, B cells, myeloid cells and keratinocytes upon exposure to an appropriate stimulus [16]. Previous studies have shown increased IL-6 production in lesional skin [7, 20] and a high level of IL-6 in plasma/serum of patients with psoriasis [5, 7]. Moreover, medical treatments including biological

agents reduce the level of IL-6 in the skin lesions [6, 7] and blood of psoriasis patients [4, 21, 24]. Therefore, IL-6 is thought to be an objective parameter correlating with clinical severity, activity, and efficacy of treatment for this condition [4–6].

Until recently, IFN-γ-producing T helper (Th) 1 cells were considered to be the main pathogenic cells in psoriasis. Recently, however, there has been increasing awareness that a new distinct lineage of $CD4^+$ Th cells, known as Th17 cells, which produce IL-17A, IL-17F, and IL-22, may play an essential role in psoriasis [2, 30]. It has been demonstrated that members of the IL-17 cytokine family (IL-17A-F) appear to show very distinct expression and are likely to have a distinct biological role [10, 13, 29].

With respect to other inflammatory conditions, Zrioual et al. [32] reported that IL-17F was specifically expressed in human rheumatoid arthritis synoviocytes, and the expression of IL-17F was higher than that of IL-17A in RA synovial tissues. As well, in asthma patients, an increase in mRNA and protein expression for IL-17F correlated with severe asthma when compared to milder disease and control groups [1]. Recent studies have demonstrated that *IL-17F* mRNA had significantly higher expression in lesional skin than in non-lesional skin of psoriasis patients [28]. In addition, IL-17F protein levels in the serum of a psoriasis (-like) mouse model have shown to be extremely elevated [18]. We previously demonstrated that IL-17F acts as a selective neutrophil attractant in psoriasis [26], although its exact role in skin disease has not been fully elucidated. To gain further understanding of the function of IL-17F, its role in normal human epidermal keratinocytes (NHEKs) and mouse skin via IL-6 production was examined. Furthermore, the expression of IL-17F by CD4-positive inflammatory cells in skin samples from psoriasis patients was examined by immunohistochemistry.

Materials and methods

All experiments were conducted in accordance with the Declaration of Helsinki Principles.

Cell culture and recombinant human IL-17F

Normal human epidermal keratinocytes (NHEKs) were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD) and used between passages 2 and 3. The cells were maintained in accordance with the supplier's protocols. NHEKs were grown using a KGM®-2 Bullet Kit® (Cambrex). Recombinant human (rh) IL-17F was generated as reported previously [12]. Endotoxin levels were tested by using Kinetic-QCL Chromogenic Limulus amebocyte lysate (Bio Whittaker, Walkersville, MD), but were undetectable. The cells were treated with IL-17F, and with the same volume of PBS as a control, for various time periods.

Animals

Female BALB/c mice were obtained from the Sankyo Laboratory (Saitama, Japan) and used at 8–10 weeks of age. Five mice were used in each experimental group, and each experiment was repeated at least 3 times. The animal protocol was approved by the institutional Animal Care and Use Committee of Showa University.

Treatment with IL-17F

To examine the effect of local administration of IL-17F on mouse skin, we injected recombinant mouse (rm) IL-17F (100 ng in 50 μl of PBS) (R&D Systems) intradermally into one ear of each mouse using a 30-gauge needle. An equal volume of PBS was injected into the ear of each mouse in the control group. The mice were anesthetized with pentobarbital sodium (50 mg/kg IP; Abbott Laboratories, IL).

Skin biopsy

We retrospectively reviewed skin biopsies from 5 patients who had been diagnosed as having psoriasis vulgaris by both dermatologists and pathologists between 2003 and 2008. All the patients had been referred to Showa University Hospital. Oval-shaped skin biopsy samples had been obtained from the lesional area including the adjacent normal skin. Each sample was prepared for routine histology and stained with hematoxylin and eosin and for immunohistochemistry. All subjects were unrelated Japanese individuals and gave written informed consent for use of their samples. The study was approved by the Ethics Committee of Showa University School of Medicine.

Immunohistochemical examination

Formalin-fixed, paraffin-embedded sections were used for examination in this study. For immunohistochemistry, a rabbit polyclonal antibody against IL-17F (IgG, MBL, Nagoya, Japan) was used as the primary antibody. Control rabbit polyclonal IgG was purchased from Santa Cruz Biotechnology, CA. A series of preliminary experiments was performed to optimize the antigen retrieval system for immunostaining of IL-17F. Heat pretreatment was performed by soaking the skin sections in 10 mM citrate buffer (pH 7.0) with surfactant and heating at 98°C for 45 min. Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide for 10 min. The sections were incubated with the primary antibody overnight at 4°C,

followed by addition of anti-rabbit secondary antibodies. Immunoreactivity was detected with Histofine Simple Stain MAX-PO (Nichirei Co., Tokyo, Japan) reagent for 30 min at room temperature. Immunocomplexes were visualized by reaction with 3,3 diaminobenzidine (DAB, DOJINDO, Kumamoto, Japan), and the sections were counterstained with Carazzi's hematoxylin.

Quantitative reverse-transcription and real-time polymerase chain reaction

Total RNA was extracted using an acid guanidinium thiocyanate–phenol–chloroform method from 1×10^6 NHEKs cells at 4 and 24 h after stimulation with 100 ng/ml IL-17F and the same volume of PBS as a control. cDNAs were synthesized from isolated RNA templates with a High-Capacity cDNA Archive Kit (Applied Biosystems, Tokyo, Japan). Pre-designed TaqMan probe sets for IL-6 were purchased from Applied Biosystems. Each probe has a fluorescent reporter dye (FAM) linked to its 5′-end, and a downstream quencher dye (TAMRA) linked to its 3′-end. We used a TaqMan Ribosomal RNA probe, labeled with a fluorescent reporter dye (VIC), as an internal control. Each reaction was performed in a 25-ml volume containing 2× Universal Master Mix (Applied Biosystems), primers, labeled probes, and 50 ng cDNA. Amplification conditions consisted of 40 cycles of 95°C for 15 s and 60°C for 1 min after incubation at 95°C for 10 min. Amplification and fluorescence measurements were carried out during the elongation step with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

Ear tissue samples from the IL-17F-treated and PBS control mice were collected at 24 h after intradermal injection, and total RNA was isolated from each ear specimen by the acid guanidinium thiocyanate–phenol–chloroform method. Primer and target-specific probes for mouse-IL-6 were purchased as predevelopment reagents from Applied Biosystems. Data are shown as fold induction relative to control cells treated with PBS.

Levels of IL-6 protein

IL-6 protein levels in the collected supernatants of stimulated NHEKs were determined with a commercially available ELISA kit (R&D Systems) in accordance with the manufacturer's instructions. Cell supernatants were harvested from cultures in the presence (or absence) of 100 ng/ml rh IL-17F, 100 ng/ml rh IL-17A or 100 ng/ml rh TNF-α. Comparisons were made to the same volume of PBS at 4, 24 or 48 h after stimulation. To account for synergistic cytokine signaling, the effect of IL-17 in combination with TNF-α was also examined. Confirmation of IL-6 being produced as a downstream effector of the IL-17 pathway was also performed using inhibitor to block IL-17 signaling. IL-17F utilizes IL-17RA and IL-17RC as its receptors [25]. An rh IL-17R/fraction crystallizable (Fc) Chimera (R&D Systems) was used to effectively inhibit signaling through IL-17RA. NHEKs were incubated in the presence (or absence) of 1.0 μg/mL IL-17R/Fc chimera with 100 ng/ml IL-17F. IL-6 production in NHEKs was examined 48 h after stimulation. The amount of secreted IL-6 was determined by ELISA and the inhibition rate by IL-17R/Fc chimera was calculated as a % inhibition of IL-17F-induced IL-6 protein level in the supernatants.

Data analysis

The statistical significance of differences was determined by analysis of variance (ANOVA). Data are expressed as the mean ± standard error of the mean (SEM) from independent experiments. Any difference with a p-value of <0.05 was considered significant. When ANOVA indicated a significant difference, the Scheffe F-test was used to determine the difference between groups.

Results

IL-6 gene expression in NHEKs induced by IL-17F

To examine the expression of the *IL-6* gene in NHEKs, we investigated whether IL-17F increased the expression of mRNA for *IL-6* using quantitative reverse transcription real-time PCR. The expression of *IL-6* mRNA stimulated by IL-17F at 4 h was 17-fold and 288-fold higher at 24 h than that in the PBS control group, respectively (Table 1).

IL-17F induces IL-6 production by NHEKs

We next investigated whether IL-17F induced IL-6 protein production by keratinocytes. Cell supernatants were harvested from cultures in the presence (or absence) of

Table 1 *IL-6* gene expression induced by IL-17F in NHEKs

Time after stimulation (h)	Group	IL-6 fold induction
4	PBS control group	1 ± 0.016
	IL-17F treated group	16.979 ± 3.438*
24	PBS control group	1 ± 0.287
	IL-17F treated group	288.469 ± 80.592*

All values, normalized with ribosomal RNA expression, are expressed as the fold induction after IL-17F stimulation compared with PBS control. Results represent mean ± SEM from at least three independent experiments

* $p < 0.05$ was considered significant

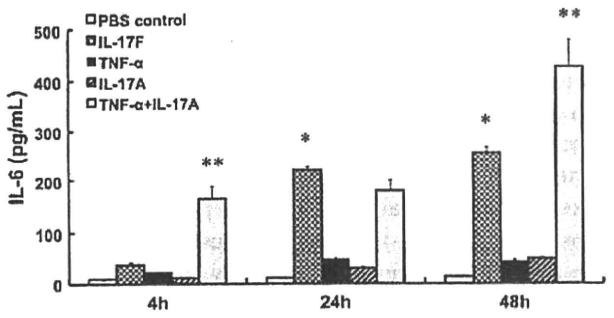

Fig. 1 Analysis of IL-6 protein production in NHEKs stimulated with IL-17F, TNF-α, IL-17A, IL-17A plus TNF-α, and PBS control. IL-6 protein release in the supernatants of stimulated NHEKs was determined by ELISA. Results represent mean ± SEM from at least three independent experiments. *The production of IL-6 in IL-17F-treated group was significantly higher ($p < 0.05$) compared with TNF-α, IL-17A, and PBS-treated groups. **The production of IL-6 in TNF-α plus IL-17A-treated group was significantly higher ($p < 0.05$) compared with IL-17F, TNF-α, IL-17A, and PBS-treated groups

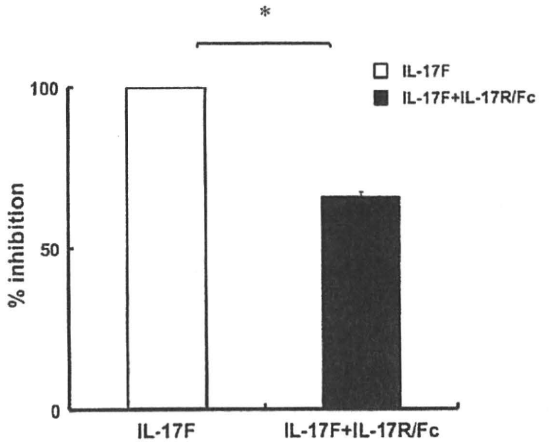

Fig. 2 Inhibition of IL-17F-induced IL-6 production in NHEKs by IL-17R/Fc chimera. IL-6 protein release in the supernatants of stimulated NHEKs was determined by ELISA. The % inhibition of IL-6 production by IL-17R/Fc chimera was calculated. Results represent mean ± SEM from three independent experiments. *$p < 0.05$ was considered significant

100 ng/ml IL-17F, 100 ng/ml IL-17A, 100 ng/ml TNF-α, or the same volume of PBS, 4, 24 or 48 h after stimulation. IL-17F elicited a time-dependent increase in the level of IL-6 protein from 4 to 48 h compared to TNF-α, IL-17A, and the PBS control (Fig. 1). Thus, the results indicated that IL-17F is a strong inducer of IL-6 in keratinocytes. To study the cooperative effect of IL-17A and TNF-α, we examined the production of IL-6 stimulated with IL-17A in combination with TNF-α. The amount of IL-6 stimulated by IL-17A plus TNF-α at 4 h and 48 h was significantly higher than by IL-17F, TNF-α, IL-17A, and PBS alone (Fig. 1). This result is in agreement with recent observations obtained using human RA synoviocytes [32].

Inhibition of IL-17F-induced IL-6 production in NHEKs by IL-17R/Fc Chimera

The receptor for IL-17F is the heterodimeric complex of IL-17RA and IL-17RC [25]. Therefore, we examined whether IL-17F-induced IL-6 was attenuated by IL-17R/Fc chimera. The production of IL-6 was 35% attenuated by IL-17R/Fc chimera ($p < 0.05$) (Fig. 2).

IL-6 gene expression in mouse skin induced by IL-17F

To further confirm expression of the *IL-6* gene, an in vivo experiment was performed. We examined whether IL-17F increased the expression of mRNA for *IL-6* after IL-17F injection in mouse skin using quantitative RT real-time PCR. The expression of *IL-6* mRNA 24 h after injection was 3.2-fold higher than that in the control group (Fig. 3).

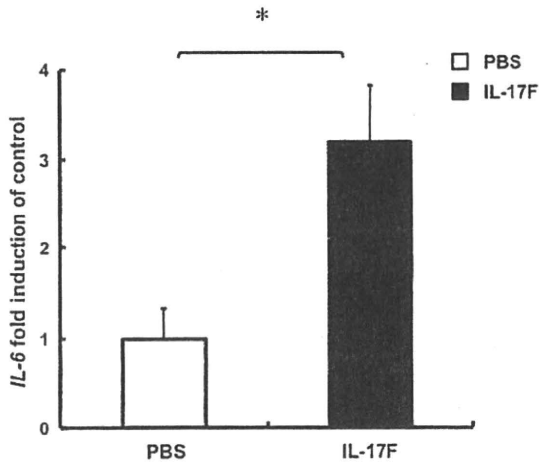

Fig. 3 *IL-6* gene expression induced by IL-17F in mouse skin. The expression of *IL-6* mRNA was quantified with real-time RT–PCR. All values, normalized with 18SrRNA expression, are expressed as the fold induction compared with PBS control. Results represent mean ± SEM from three independent experiments. *$p < 0.05$ was considered significant

Expression of IL-17F by $CD4^+$ T cells in the skin of psoriasis patients

To examine the IL-17F expression in skin disease, we examined five skin biopsy samples from psoriasis vulgaris patients for expression of CD4, CD8, IL-17F, and an isotype control, by immunohistochemical staining. Each staining was performed using step sections from the same samples. In psoriatic lesional skin, most of the inflammatory cells in the papillary areas in the upper dermis were positive for CD4 (Fig. 4a). A small number of inflammatory

cells in the upper dermis were positive for CD8 (Fig. 4b). These findings were consistent with the general concept of psoriasis as a T-cell-mediated disorder, with mainly $CD4^+$ T-lymphocyte subsets present within the dermis [8, 19]. Immunohistochemical staining for IL-17F suggested that many of the $CD4^+$ cells were also positive for IL-17F using skip sections of psoriatic lesional skin (Fig. 4c, e). In non-lesional skin from the same psoriasis patient, the inflammatory cells in the upper dermis were positive for CD4 (Fig. 4h), whereas in the step sections few were positive for IL-17F, or were mostly negative (Fig. 4f). These findings indicated that IL-17F was secreted by $CD4^+$ cells in psoriatic lesions, whereas little was secreted in non-lesional skin from the same patient. These results were in accord with our previous observation that IL-17F production was significantly higher in lesional skin than in non-lesional skin, although a low level of IL-17F was detected in psoriatic non-lesional skin by the ELISA method [26].

Discussion

In this study we demonstrated that IL-17F is able to induce IL-6 production both in NHEKs and in mouse skin. As well, we showed that $CD4^+$ T cells in skin from psoriasis patients express IL-17F. It is noteworthy that recent studies have demonstrated increased expression of IL-6 in IL-17F-over-expressing mice, thus further supporting a role of IL-17F in the induction of IL-6 [9, 22, 29]. Unstimulated NHEKs produce minimal levels of IL-6. In contrast, NHEKs are capable of producing IL-6 mRNA and protein when exposed to certain stimuli [16]. The present study demonstrated that the amounts of IL-6 stimulated by IL-17F were much higher than those stimulated by TNF-α or IL-17A. We showed for the first time that IL-17F is a crucial cytokine that induces a high amount of IL-6 in NHEKs. However, its regulatory mechanisms remain to be clarified. Until recently, IL-1β and TNF-α have been considered the potent inducers of IL-6 [16]. Interestingly, IL-17A is able to induce IL-1β and TNF-α production [11]. Therefore, we could not fully exclude the possibility that IL-17F induces the production of IL-6 via IL-1β and TNF-α. Further study is needed in the future to clarify this regulatory interaction. Importantly, we did demonstrate that synergistic effects of IL-17A and TNF-α were capable of upregulating IL-6 as well. This finding was consistent with the known ability of IL-17A to cooperatively interact with other pro-inflammatory cytokines in various tissues [14, 32]. To further delineate the signaling mechanism of IL-17F for upregulating IL-6, we were able to demonstrate partial inhibition of IL-17F-induced IL-6 production by IL-17R/Fc chimera in keratinocytes.

A previous study has demonstrated that injection of exogenous IL-12 or tumor necrosis factor in mouse back skin failed to induce marked inflammation [3]. In the present study, mouse skin that had been injected with IL-17F expressed a higher level of IL-6 than that injected with PBS as a control. We recently demonstrated that IL-17F induced remarkable infiltration of neutrophils in the mouse ear [26]. Furthermore, the IL-23 injections caused acanthosis with a mixed infiltrate containing Th17 cells [31]. These observations suggest that IL-17F is able to cause dermal inflammation and induces various inflammatory cytokines including IL-6 in vivo as well as in vitro.

Generally, psoriasis is regarded as a T-cell-mediated disorder, with mainly $CD4^+$ T-lymphocyte subsets present within the dermis [8, 19]. Consistent with this notion, our immunohistochemical examination of skin samples from psoriasis patients' demonstrated that many of the inflammatory cells in the dermis were CD4-positive, whereas few were CD8-positive. Furthermore, in step sections from the same samples, almost all of the $CD4^+$ cells were IL-17F-positive. On the other hand, in non-lesional skin from the same psoriasis patient, the inflammatory cells in the dermis were positive for CD4, but few were positive for IL-17F, and most were negative. These results support the notion that a novel subset of $CD4^+$ cells characterized by IL-17F production may play a crucial role in maintaining inflammation in psoriatic lesions.

IL-6 is produced in a regulated manner by keratinocytes, fibroblasts and vascular endothelial cells as well as by leukocytes infiltrating the skin [7, 16]. IL-6 can stimulate the proliferation of human keratinocytes under some conditions [7, 16]. Psoriasis is one of several inflammatory skin diseases in which elevated expression of IL-6 has been described [7, 27]. Lesional skin from psoriasis patients expresses a high level of IL-6 in comparison with non-lesional skin and with plaques remaining after treatment [7, 20]. Increased levels of IL-6 have also been reported in the plasma and serum of patients with active psoriasis [4, 5, 7]. Therefore, IL-6 could directly contribute to the epidermal hyperplasia seen in psoriatic epithelium as well as affecting the function of dermal inflammatory cells. Moreover, it has been demonstrated that IL-6 induces Th17 cell differentiation in humans [10]. Taken together, the data suggest that IL-17F-induced IL-6 produced by keratinocytes promotes the development of Th17 cells as an autocrine regulator. Thus, the IL-17F/IL-6 axis may enhance inflammation of the lesional skin in psoriasis.

In conclusion, this study has revealed that IL-17F secreted by $CD4^+$ T cells likely contributes to the inflammatory milieu of psoriasis via induction of IL-6 by keratinocytes. We have confirmed a functional linkage between IL-17F and IL-6 in cutaneous inflammation to further support the regulatory role they may play in psoriasis. Recent studies of Th17 cells have already led to successful therapeutic strategies for psoriasis [15, 17, 23]. Future research

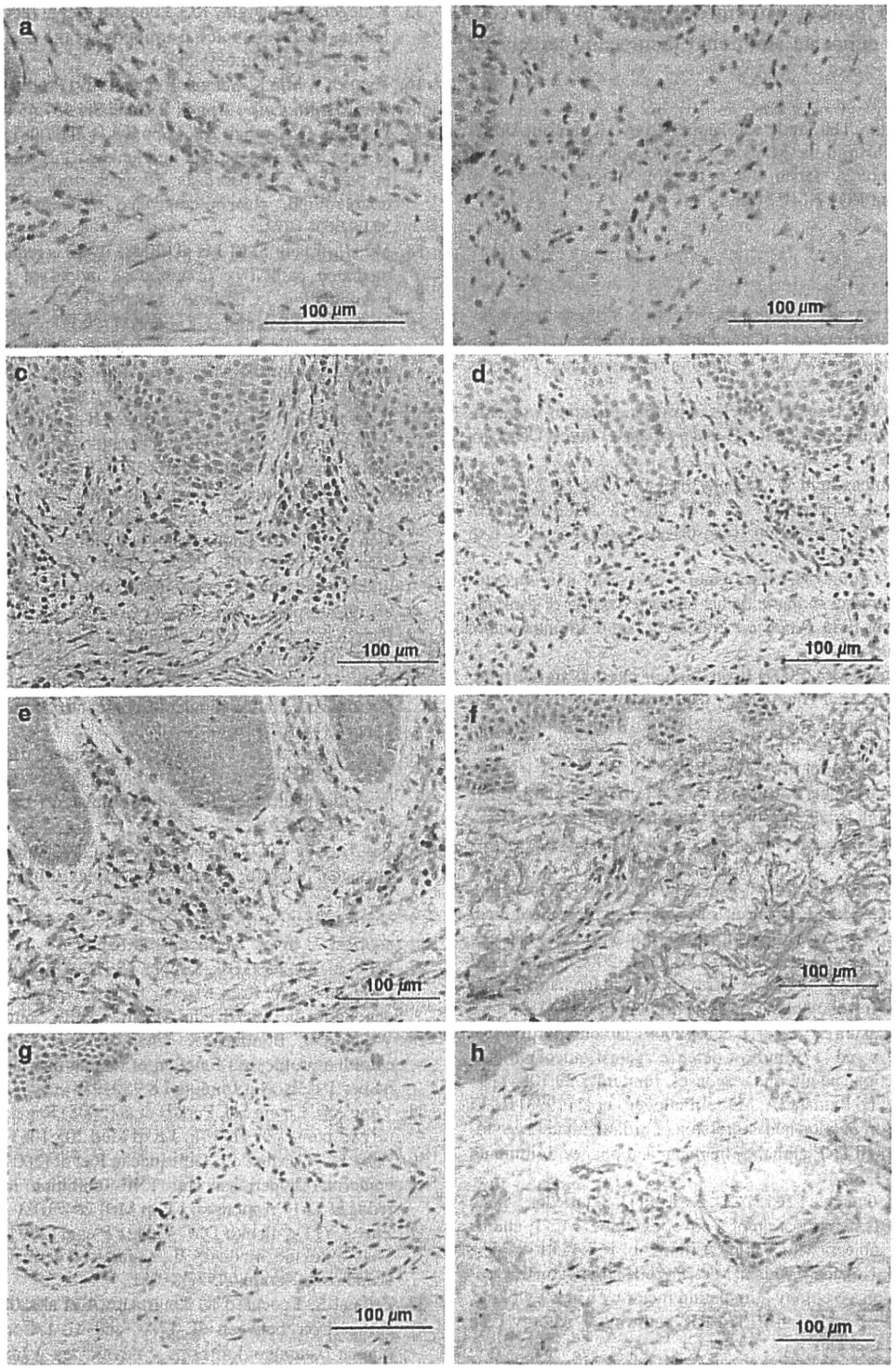

Fig. 4 Expression of IL-17F on $CD4^+$ T cells in the skin of psoriasis patients. Representative results from immunohistochemical staining in the lesional skin and non-lesional skin of psoriasis patients. **a** In psoriatic lesional skin, most of the inflammatory cells in the upper dermis are positive for CD4 staining. **b** A small number of inflammatory cells in the upper dermis are positive for CD8. Immunohistochemical staining for IL-17F using step sections showed that many of the $CD4^+$ cells (**e**) were also positive for IL-17F (**c**) in psoriatic lesional skin. In non-lesional skin from the same psoriasis patient, the inflammatory cells in the upper dermis were positive for CD4 (**h**), but few were positive for IL-17F and most were negative using the step sections (**f**). **d**, **g** Staining of the same plaque with the isotype control of IL-17F (rabbit polyclonal IgG)

will continue to focus on cytokines produced by Th17 cells and attempt to delineate additional therapeutic targets for psoriasis.

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Erythema multiforme major putatively induced by dihydrocodeine phosphate

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Dihydrocodeine phosphate is used in many over-the-counter (OTC) cold medicines in Japan.[1] We report a case of erythema multiforme major (EMM) putatively induced by this drug.

A 74-year-old woman presented with pruritic lesions in May 2005. Her medical history included osteoporosis. She had previously attended the urology department, where urinalysis revealed bacilluria and a urine culture was positive for *Escherichia coli*, thus the patient had been diagnosed as having cystitis. She had been given amoxicillin 750 mg/day for 1 week, starting 12 days before her presentation to us. She later developed pharyngodynia and fever of 38.5 °C, and she took a tablet of an over-the-counter cold medicine (Pabron S®; Taisho Pharmaceutical Co. Ltd, Tokyo, Japan) containing bromhexine chloride as the active ingredient, 4 days before presentation (2 days after finishing the course of antibiotics). She had previously taken the medicine several times and she was on no other medications. That night she developed a generalized rash. She presented to another hospital, which prescribed systemic corticosteroids (prednisolone 20 mg/day) for 3 days, but the rash persisted, and she then came to our dermatology department.

On physical examination, we found oedematous erythema on both periorbital regions. The patient had bilateral conjunctival hyperaemia and erosive lesions on both angles of the mouth. Targetoid patches, dark-red or purpuric in colour, were present on the trunk and limbs (Fig. 1). About two-thirds of the body surface area was affected by the rash.

Laboratory investigations gave normal results for white cell blood count and differential cell count, and for a biochemical screen. Anti-streptolysin O (ASO) antibody and antistreptokinase (ASK) antibody levels were normal, and tests were negative for *Mycoplasma* antibody. Serological tests (ELISA) for herpes simplex virus, Epstein–Barr virus and cytomegalovirus antibodies also gave negative results. Results of tests carried out on day 14 after presentation showed no IgM antibody increase compared with day 1.

A biopsy specimen was taken from an erythematous lesion on the left arm, and exocytosis of lymphocytes and liquefaction of the basal cell layer in the epidermis were seen. Oedemal and perivascular infiltration of lymphocytes were seen in the upper dermis. We diagnosed EMM on the basis of the morphology and the clinical and histological findings.

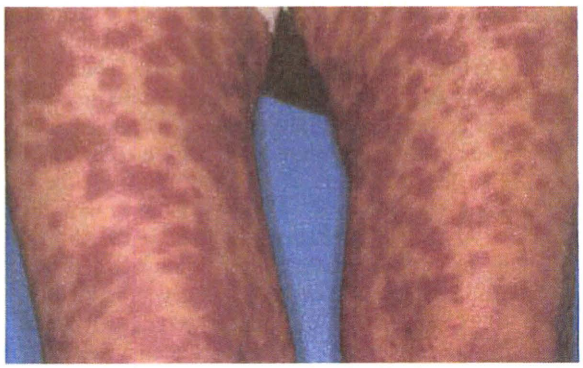

Figure 1 Targetoid patches, dark-red or purpuric in colour, on the limbs.

The patient was admitted to hospital and started on topical ophthalmic treatment (0.5% levofloxacin eyedrops and 0.1% fluorometholone eyedrops). Predonisolone was given: 20 mg/day for 4 days and 10 mg/day for 3 days. The patient was discharged on day 7, when most cutaneous lesions had subsided except for hyperpigmentation; the conjunctival hyperaemia also disappeared.

Patch testing was performed with 10% Pabron S® and 10% amoxicillin 16 days after the patient was discharged. The results were assessed using the International Contact Dermatitis Research Group (ICDRG) scoring system at 2 and 3 days after application.[2] A positive reaction to Pabron S® and a negative reaction to amoxicillin were seen. One month later, another test was performed using the ingredients of the Pabron S® tablet. A positive reaction to a dilution of 10% (1% dihydrocodeine phosphate) was seen; all the other ingredients (10% acetaminophen, 10% bromhexine chloride, 10% noscapine, 10% DL-methylephedrine hydrochloride, 10% arbinoxamine maleate, 10% caffeine anhydride, 10% bisibuthiamine, 10% lysozyme chloride) and the control (hydrophilic petrolatum) gave negative reactions at both time points.

In this case, we cannot exclude the possibility that the pharyngodynia and fever were the initial symptoms of EMM. As the ASO, ASK and *Mycoplasma* antibody titres were measured 4 days after the onset of the sore throat, the results may be false negatives. However, we speculate that these symptoms were caused by an upper respiratory infection as they had disappeared at her initial visit and EMM generally produces a lower fever. Although there are several causes of EMM, we consider that a drug reaction was the most likely in this case; no findings suggested herpesvirus or *Mycoplasma* infection, collagen diseases, or malignancy. Dihydrocodeine phosphate, a methymorphinan derivative, has an analgesic and antitussive action,[1] and is a common ingredient in OTC cold medicines in Japan;[3] the Pabron S® tablet contained 8 mg of the drug.

Ten cases of dihydrocodeine phosphate drug eruptions have been reported in Japan:[4] three cases of exanthematous reactions, and one each of urticaria, eczematous reaction, fixed drug eruption, EM, Stevens–Johnson's syndrome, erythroderma and unknown.[4] Of the 10 cases, four had a positive patch test reaction. We consider that 10% (1% dihydrocodeine phosphate) may not be an irritant as we have encountered seven patients with drug eruption who had a negative reaction to it. Although patch testing in drug eruptions can be valuable, this is not always the case; for instance, it has been reported that only 20.9% of EM cases had a positive reaction.[5] However, positive results can be useful and relevant to patient management.[6] In patients with drug eruptions to a cold medicine such as ours, it is important to assay not only the medication itself, but also its component ingredients, as it is likely that these may also occur in similar products.

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Y. Matsuzawa, S. Fujishima, T. Nakada and M. Iijima
Department of Dermatology, Showa University School of Medicine, 1-5-8, Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan
E-mail: tokio@med.showa-u.ac.jp
Conflict of interest: none declared.
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Primary cutaneous lymphoma associated with Epstein–Barr virus and azathioprine therapy

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A 67-year-old man with a 2-year history of dermatomyositis, controlled with prednisolone (7.5–15 mg daily) and azathioprine (150–175 mg daily), presented with a spontaneous, painless, ulcerating plaque on the upper back (Fig. 1). He was systemically well apart from fatigue, which was of long standing. He had no lymphadenopathy or organomegaly.

Laboratory investigations including full blood count, erythrocyte sedimentation rate, levels of lactate dehydrogenase and C-reactive protein, and renal, liver and bone profile gave normal results.

Histological examination of a biopsy taken from the ulcer edge found a denuded epidermis and a superficial and deep dermal infiltrate composed of large lymphoid cells with large nucleoli and a small amount of pale cytoplasm with admixed smaller lymphoid cells (Fig. 2). Immunohistochemical examination revealed a large cell population positive for CD30 (Fig. 2), CD20 and CD79a, and negative for CD3, CD4, CD8, CD15 and ALK1. Histology was consistent with a diffuse large B-cell lymphoma. Immunohistochemistry for Epstein–Barr virus (EBV) was positive. PCR for EBV on DNA extracted from the formalin-fixed skin

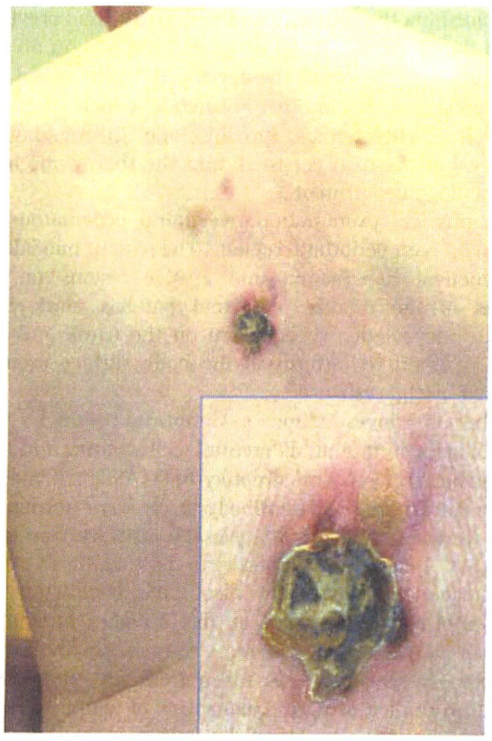

Figure 1 Spontaneous, painless ulcer on the upper mid-back.

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LETTER TO THE EDITOR

Angiosarcoma (Stewart–Treves syndrome): Palliative role of Mohs' ointment

Dear Editor,

A 79-year-old woman with cutaneous lesions of the left upper extremity was examined in August 2008. Family history was as follows: mother, colon cancer; older sister, gastric cancer; and older brother, colon cancer. Past medical history included breast cancer in 1998, gastric cancer in 2005 and renal cancer in 2006. She had undergone left mastectomy for breast cancer 11 years earlier. Six months post-surgery, edema appeared on the left upper limb. Cutaneous lesions had developed on the edematous extremity in the prior 2 months. Physical examination revealed overt edema and scattered lesions of cyanotic patches and dark reddish nodes on the left upper extremity. We diagnosed angiosarcoma (Stewart–Treves syndrome) on the basis of history and morphology. Although we proposed several options of therapy, such as taxane, the patient refused them. In the following 3 months, the lesions enlarged and increased in number. A biopsy was performed from a dark reddish nodule on the extensor aspect of the left forearm. Histopathological examination of the biopsy specimen demonstrated proliferation of atypical cells in the dermis and subcutaneous tissue. The tumor cells were atypical endothelial cells and vascular spaces were lined by these cells. These tumor cells were $CD31^+$ and $CD34^+$. Because systemic therapy was not believed useful, symptomatic therapy was initiated. She was admitted for anemia: hemoglobin 7.3 g/dL, caused by external bleeding 5 months after her initial visit (Fig. 1). Because compression and external application with thrombin were ineffective for management of external bleeding, Mohs' ointment[1] was employed 1 month post-admission. Mohs' ointment was applied for lesions with 1 mm thickness once daily. External bleeding from the lesions was decreased (Fig. 2), and spontaneous pain decreased. However, enlargement of the lesions and bleeding from larger lesions could not be prevented. She died of respiratory failure due to metastases 11 weeks post-admission (7 months after the initial visit).

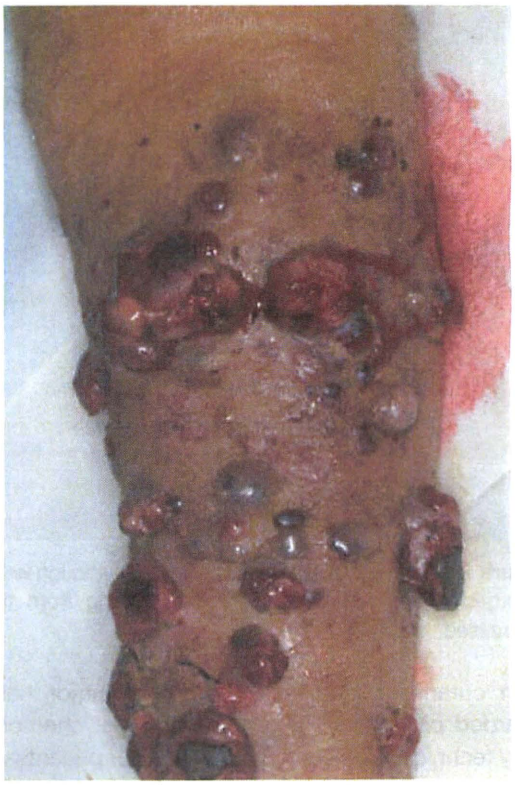

Figure 1. Dark reddish nodes and bleeding from these lesions on the left forearm.

Stewart–Treves syndrome occurs in 0.5% of patients who survive mastectomy for more than 5 years.[2] The mean age at onset of angiosarcoma is 62 years, and the mean interval between mastectomy and tumor onset is 10.5 years.[2] In our case, the onset was 11 years post-mastectomy. Although recent studies report that taxane was effective in patients

Correspondence: Tokio Nakada, M.D., Department of Dermatology, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666 Japan. Email: tokio@med.showa-u.ac.jp

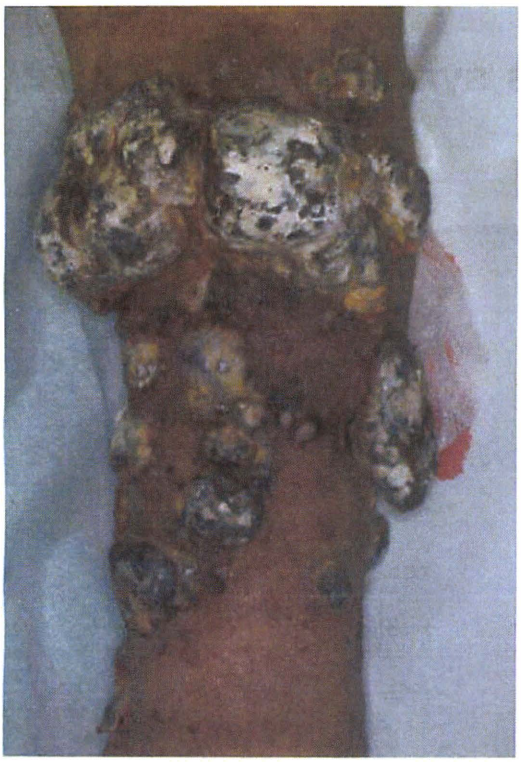

Figure 2. Post-Mohs' ointment application. Although lesions enlarged and increased in number, bleeding from them decreased.

with cutaneous angiosarcoma,[3] this tumor has a guarded prognosis like our case. Mohs' chemosurgery technique has been used mainly for patients with skin cancer (e.g. basal cell carcinoma).[1] In this case, Mohs' paste was a palliative therapy mainly for management of external bleeding. Like our case, it was used for bleeding from advanced malignancies: head and neck cancer,[4] breast cancer revealing unresectable skin lesions,[5–7] and malignant melanoma.[8] In our case, although pain score on the admission day was 6 by using a Visual Analog Scale (VAS: range 0–10, in which lower numbers indicate less pain), the score reduced to less than 5 post Mohs' technique. Hence, it played a role in the patient's quality of life.

Hirokazu UNO, Miki SASAKI, Kurato OSAMURA, Shinpei OHTOSHI, Tokio NAKADA, Masafumi IIJIMA

Department of Dermatology, Showa University School of Medicine, Tokyo, Japan

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Genome-wide association study identifies *HLA-A*3101* allele as a genetic risk factor for carbamazepine-induced cutaneous adverse drug reactions in Japanese population

Takeshi Ozeki[1], Taisei Mushiroda[1], Amara Yowang[1], Atsushi Takahashi[2], Michiaki Kubo[3], Yuji Shirakata[4], Zenro Ikezawa[5], Masafumi Iijima[6], Tetsuo Shiohara[7], Koji Hashimoto[4], Naoyuki Kamatani[2] and Yusuke Nakamura[1,8,*]

[1]Research Group for Pharmacogenomics, [2]Research Group for Medical Informatics and [3]Research Group for Genotyping, RIKEN Center for Genomic Medicine, Yokohama 230-0045, Japan, [4]Department of Dermatology, Ehime University Graduate School of Medicine, Ehime 791-0295, Japan, [5]Department of Dermatology, Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan, [6]Department of Dermatology, Showa University School of Medicine, Tokyo 142-8555, Japan, [7]Department of Dermatology, Kyorin University School of Medicine, Tokyo 181-8611, Japan and [8]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

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An anticonvulsant, carbamazepine (CBZ), is known to show incidences of cutaneous adverse drug reactions (cADRs) including Stevens–Johnson syndrome (SJS), toxic epidermal necrolysis (TEN) and drug-induced hypersensitivity syndrome (DIHS). To identify a gene(s) susceptible to CBZ-induced cADRs, we conducted a genome-wide association study (GWAS) in 53 subjects with the CBZ-induced cADRs, including SJS, TEN and DIHS, and 882 subjects of a general population in Japan. Among the single nucleotide polymorphisms (SNPs) analyzed in the GWAS, 12 SNPs showed significant association with CBZ-induced cADRs, and rs1633021 showed the smallest *P*-value for association with CBZ-induced cADRs ($P = 1.18 \times 10^{-13}$). These SNPs were located within a 430 kb linkage disequilibrium block on chromosome 6p21.33, including the *HLA-A* locus. Thus, we genotyped the individual *HLA-A* alleles in 61 cases and 376 patients who showed no cADRs by administration of CBZ (CBZ-tolerant controls) and found that *HLA-A*3101* was present in 60.7% (37/61) of the patients with CBZ-induced cADRs, but in only 12.5% (47/376) of the CBZ-tolerant controls (odds ratio = 10.8, 95% confidence interval 5.9–19.6, $P = 3.64 \times 10^{-15}$), implying that this allele has the 60.7% sensitivity and 87.5% specificity when we apply *HLA-A*3101* as a risk predictor for CBZ-induced cADRs. Although DIHS is clinically distinguished from SJS and TEN, our data presented here have indicated that they share a common genetic factor as well as a common pathophysiological mechanism. Our findings should provide useful information for making a decision of individualized medication of anticonvulsants.

INTRODUCTION

Cutaneous adverse drug reactions (cADRs) characterized by acute inflammatory reaction of skin and mucous membranes are dose-independent, unpredictable and sometimes life-threatening. Manifestations range from a mild erythematous maculopapular rash [maculopapular eruption (MPE)], a self-limited, exanthematous, cutaneous variant with minimal oral

*To whom correspondence should be addressed at: Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Tel: +81 354495372; Fax: +81 354495433; Email: yusuke@ims.u-tokyo.ac.jp