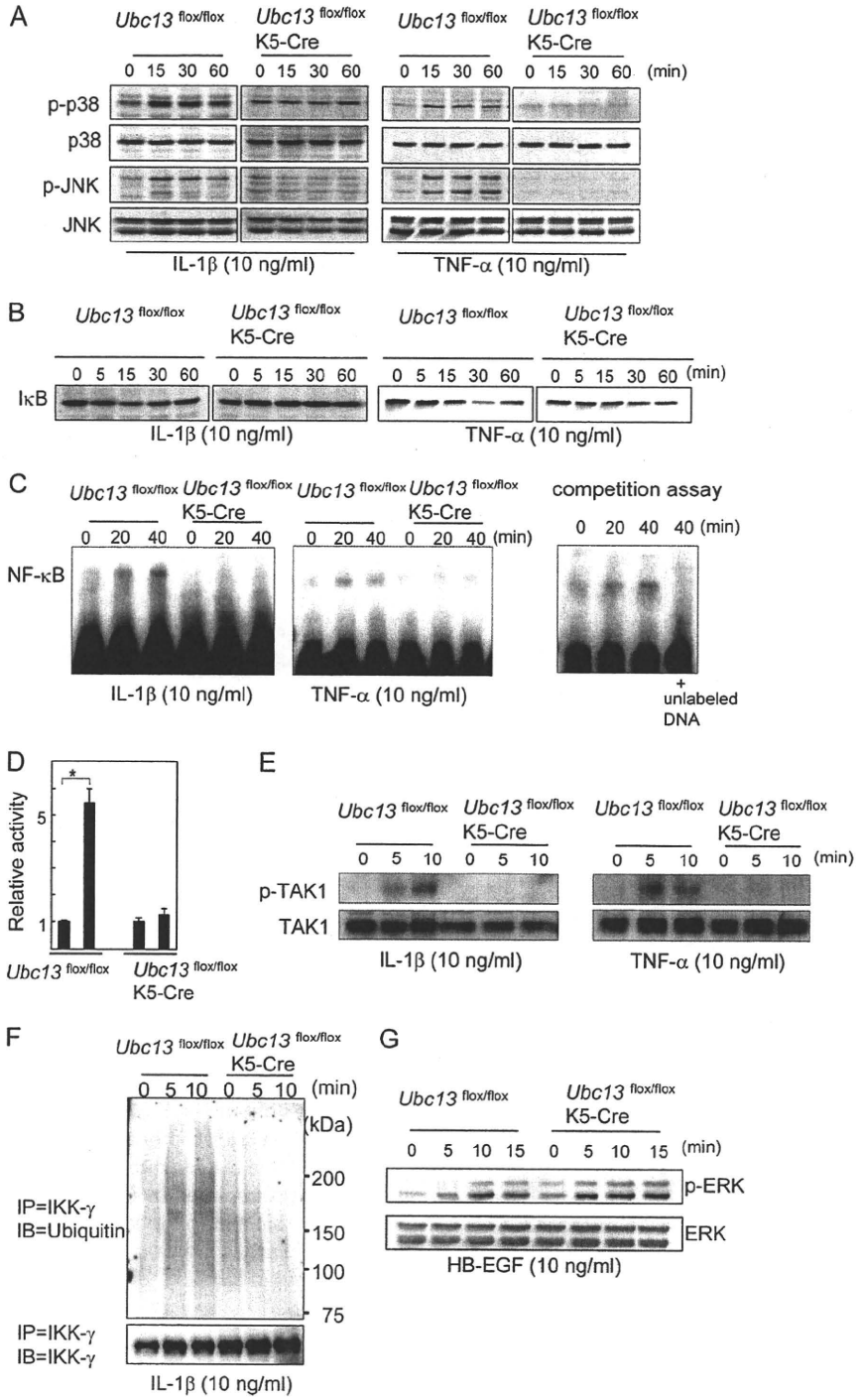

FIGURE 6. **Impaired activation of p38, JNK, and NF-κB in Ubc13-deficient keratinocytes.** Freshly isolated mouse keratinocytes were stimulated with IL-1β (10 ng/ml), TNF-α (10 ng/ml) (*A–E*), or HB-EGF (10 ng/ml) (*F*). Intracellular signals were then analyzed by Western blotting (*A*, *B*, *E*, and *G*), EMSA (*C*), luciferase assay (*D*), and immunoprecipitation (*F*, *IP*). *Ubc13*$^{flox/flox}$ represents the undeleted controls. *A*, phosphorylation of p38 and JNK (*p-p38* and *p-JNK*, respectively). p38 and JNK were used as standards. *B*, Western blotting of IκB. *C*, NF-κB activity was analyzed by EMSA. Protein-DNA complexes were separated and transferred to nylon membranes. In competition assay, *Ubc13*$^{flox/flox}$ keratinocytes were stimulated with IL-1β (10 ng/ml), and unlabeled probe (200-fold molar excess) was added to the sample of 40 min. The shift by IL-1β was prevented by the unlabeled probe, indicating that the shift was the result of specific protein-DNA interaction. *D*, luciferase assay. After transfection of pNFκB-TA-Luc, the keratinocytes were stimulated with IL-1β (10 ng/ml) for 24 h. The relative luciferase activity was calculated by normalizing to the level of *Renilla* luciferase activity. The data are expressed as the means ± S.E. *, $p < 0.01$. $n = 3$. *E*, phosphorylation of TAK1 (p-TAK1) was analyzed by Western blotting. TAK1 was the standard. *F*, ubiquitination of IKK-γ. The samples were first immunoprecipitated with anti-IKK-γ and then immunoblotted (*IB*) with anti-ubiquitin. In control cells, broad bands were detected at 5 and 10 min after the IL-1β stimulation. *G*, phosphorylation of ERK (ERK was the standard). These studies (*A–G*) were performed more than three times, and the representative data are shown.

phenotype is not apparent in TAK1- or IKK-γ-deficient mice at birth, the skin of our Ubc13-deficient mice was already abnormal at birth. The number of apoptotic cells in Ubc13-deficient epidermis was much lower than that in TAK1-deficient epidermis (14). Furthermore, the microabscesses seen in TAK1- or IKK-γ-deficient epidermis were not observed in our Ubc13-deficient mice. Although Ubc13 has been shown to regulate the NF-κB pathway in keratinocytes (Fig. 6), the phenotype produced by Ubc13 deficiency compared with that of TAK1 or IKK-γ deficiency indicates that the signaling controlled by Ubc13 is not identical to that of TAK1 or IKK-γ.

The role of Ubc13 and TAK1 in the activation of JNK, p38, and NF-κB varies by cell type and stimulus. TAK1 is indispensable for the activation of NF-κB and MAPK by TLRs, IL-1 receptor, and TNF receptor (1, 2). However, TAK1-deficient B cells can still activate NF-κB in response to B cell receptor stimulation (1). Thymocytes from TAK1-deficient mice display severely defective NF-κB activation in response to anti-CD3/CD28 stimulation (1, 25, 26); however, NF-κB activation is normal in the peripheral T cells of such animals (26). *In vivo* analyses of mice lacking Ubc13 in their B cells, myeloid cells, and embryonic fibroblasts have shown nearly normal NF-κB activation during BCR-, IL-1 receptor-, TLR, or CD40-mediated signal transduction (15), indicating that Ubc13 plays a minor role in these signaling pathways (15). Although NF-κB activation was modestly affected, JNK and p38 activation was impaired in Ubc13-deficient thymocytes (27). During IL-1 receptor-mediated signaling in these cell types, Ubc13 is more important for the activation of p38 and JNK than of NF-κB (27). However, in this study we found that in keratinocytes, Ubc13 is essential for the IL-1 and TNF-induced activation of the NF-κB, JNK, and p38 pathways. The impaired activation of these signaling pathways may cause the functional defect of Ubc13-deficient keratinocytes.

Ubc13 deficiency causes spontaneous cell death that is associated with decreased cIAP-2 and the activation of caspase-3 (Fig. 5*E*). Similarly, in TAK1-deficient keratinocytes, the expression of cIAP-2 is down-regulated and caspase-3 is activated, which causes TRAIL-induced cell death (22). These data suggest that the TAK1 deletion facilitates TRAIL-induced cell death by activating caspase through down-regulating cIAP (22). Therefore, it is most likely that decreased cIAP-2 enhanced the susceptibility to cell death in Ubc13-deficient keratinocytes, as well as TAK1. Because cIAP-2 is a target molecule of NF-κB, the down-regulation of cIAP-2 may be due to the impaired NF-κB pathway in Ubc13-deficient keratinocytes. This spontaneous cell death is enhanced slightly by TNF-α. Because TNF-α mRNA expression in the epidermis of $Ubc13^{flox/flox}$K5-Cre mice was decreased (Fig. 2*C*) and cell death was not blocked by anti-TNF-α antibodies (Figs. 4*D* and 5*D*), spontaneous cell death is not likely to be due to endogenous TNF-α. In studies of IKKs-deficient keratinocytes, the cells were also susceptible to TNF-induced cell death (28–30), as well as TAK1 (21). However, the effects are limited compared with the TAK1 deletion. In Ubc13-deficient keratinocytes, the cytotoxic effects of TNF are also limited (Figs. 4*D* and 5*D*). The low sensitivity to TNF in Ubc13-deficient cells may be partially due to the spontaneous cell death. Because spontaneous cell death occurs via the deletion of Ubc13 alone, susceptible cells may undergo cell death without TNF. The remaining cells may be somewhat resistant to cell death.

During embryogenesis, the NF-κB pathway is activated at the placode via binding of EdaA1 to its receptor, EdaR (31). This EdaA1/EdaR/NF-κB pathway is essential for the development of ectodermal appendages such as hair follicles, teeth, and sweat glands (32, 33). In addition, mutations of these genes can cause reduced or absent ectodermal appendages. In $Ubc13^{flox/flox}$ K5-Cre mice, the skin was abnormally shiny and smooth. Because the NF-κB pathway is impaired in these mice, this phenotype may be partially due to the anomaly of ectodermal appendages; however, this point should be studied further.

Because the NF-κB pathway regulates pro-inflammatory responses, the disruption of this pathway likely has a negative effect on epithelial inflammation. However, the deletion of TAK1, IKK-β, or IKK-γ results in severe inflammation (abscess formation) in the skin (10, 11, 14, 19). Similarly, a lack of NF-κB signaling caused by the conditional ablation of IKKγ or IKKα and IKKβ in the intestinal epithelium causes severe chronic intestinal inflammation in mice (34). Continuous NF-κB activation at the basal level may be required to maintain the homeostasis of the epithelium. However, the deletion of Ubc13 in epidermal keratinocytes does not cause inflammation. One possible explanation for this is the early mortality of the $Ubc13^{flox/flox}$ K5-Cre mice by postnatal day 2, before the occurrence of epidermal inflammation. In conclusion, Ubc13 in keratinocytes appears to be essential for maintaining epidermal integrity in mice.

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PPARγ mediates innate immunity by regulating the 1α,25-dihydroxyvitamin D3 induced hBD-3 and cathelicidin in human keratinocytes

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ABSTRACT

Background: Production of antimicrobial peptides (AMPs) is the primary mechanism by which skin innate immunity protects against infection. Hormonally active vitamin D3 (1α,25-dihydroxyvitamin D3; $1,25D_3$) is a vital regulator of skin innate immunity, and has been shown to increase the expression and function of AMPs.

Objective: PPARγ is a ligand-activated nuclear receptor and plays a role in keratinocyte differentiation and cutaneous homeostasis. In this study, we investigate whether $1,25D_3$-activated PPARγ signaling regulates AMP expression in keratinocytes.

Methods: Subconfluent keratinocytes were treated with $1,25D_3$ for the indicated times. The mRNA and protein levels of AMPs were detected by RT-PCR and Western blot, and the DNA binding activation of PPARγ, VDRE and AP-1 was investigated by EMSA. To examine the role of PPARγ, the recombinant adenovirus carrying a dominant-negative form of PPARγ (dn-PPARγ) was constructed and transfected into keratinocytes.

Results: We show here that $1,25D_3$ significantly enhances hBD-3 and cathelicidin expression in keratinocytes. Expression of dn-PPARγ did not affect binding to the vitamin D-responsive element (VDRE), which is crucial for cathelicidin induction by VD3; however, it did decrease $1,25D_3$ induction of both hBD-3 and cathelicidin. Inhibition of the p38, ERK, and JNK signaling pathways blocked hBD-3 expression, whereas only p38 inhibition suppressed cathelicidin induction. dn-PPARγ had no effect on ERK and JNK activity, but inhibited p38 phosphorylation and suppressed $1,25D_3$-induced AP-1 activation via effects on Fra1 and c-Fos proteins.

Conclusions: In conclusion, PPARγ regulates the $1,25D_3$-induced hBD-3 and cathelicidin expression in keratinocytes through the regulation of AP-1 and p38 activity.

1. Introduction

Epidermal keratinocytes differentiate to form a multilayered epidermis that is the primary barrier between the body and the outer environment. Despite the constant exposure of the epidermis to a wide varied of microbial pathogens, skin infections are relatively rare. The synthesis and secretion of AMPs by epithelia is recognized as an important mechanism for host defense. As effectors of innate immunity, AMPs directly kill a broad spectrum of microbes, including Gram-positive and Gram-negative bacteria as well as fungi and certain viruses. They also trigger and coordinate multiple components of the innate and adaptive immune system [1,2].

The major AMPs found in humans are cathelicidins and defensins [1,3]. Defensins are classified as α- or β-defensins based on the distribution of the cysteines and disulfide bonds. Whereas most α-defensins are produced by neutrophils, human β-defensins (hBDs) are generated mainly by epithelial tissues including the skin and respiratory tract. The three best-characterized hBDs, hBD-1, hBD-2, and hBD-3, have been detected in human skin and cultured keratinocytes. hBD-1 expression is primarily constitutive, whereas hBD-2 and hBD-3 expression is induced by cytokines, as well as growth factors, various microorganisms, and other microbial products [2,3]. In addition to hBDs, the skin epithelium also generates cathelicidins [1,2], a family of antimicrobial peptides with a highly conserved N-terminal cathelin domain and a C-terminal cationic antimicrobial domain that is

Abbreviations: AMP, antimicrobial peptide; $1,25D_3$, 1α,25-dihydroxyvitamin D3; Ax, adenovirus vector; PPAR, peroxisome proliferator-activated receptor; VDR, vitamin D receptor; VDRE, vitamin D response elements; hBD, human β-defensin.

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activated following cleavage. Although many cathelicidins have been identified in mammals, only one cathelicidin AMP, also known as hCAP18/LL-37/FALL-39, is present in humans [1,2]. The biological relevance of these AMPs in skin has been illustrated by several clinical correlations [1,2,4]. Thus, patients with atopic dermatitis lack the appropriate AMPs and are more susceptible to skin infections, whereas psoriasis patients, who rarely develop bacterial skin infections, show high epidermal AMP expression. In spite of these, the molecular mechanisms of AMP regulation in keratinocytes are poorly understood.

$1,25D_3$, the hormonally active form of vitamin D3, decreases proliferation and increases terminal differentiation of keratinocytes, and also acts as a regulator of skin immunity. In skin injury, activated vitamin D3 metabolism leads to rapid induction of genes important for microbial recognition and antimicrobial defense [5]. The genomic effects of $1,25D_3$ are mediated by its nuclear hormone receptor, the vitamin D receptor (VDR). After activation by $1,25D_3$, the VDR binds to consensus sequences known as VDREs in the promoter region of target genes, including cathelicidin and hBD-2 [6]. $1,25D_3$ induces cathelicidin and/or hBD-2 expression in keratinocytes and myeloid cells [6–9], and strengthens cell antimicrobial activity [6–8]. Topical treatment of human skin with $1,25D_3$ also enhance cathelicidin peptide expression [8], suggesting a role of vitamin D3 or its analogs as topical immune modulators [5].

PPARγ signaling regulates keratinocyte differentiation and cutaneous homeostasis, in a manner similar to other nuclear hormones such as glucocorticoids, retinoids, and vitamin D [10–12]. We have previously showed that suspension culture and $1,25D_3$ treatment of keratinocytes increases PPARγ expression and activity, which contributes to involucrin expression and keratinocyte differentiation [13,14]. Keratinocyte differentiation often upregulates innate immunity [15], but the function of PPARγ in skin innate immunity remains unclear. We investigated if $1,25D_3$-activated PPARγ signaling regulates AMP expression in keratinocytes, and demonstrated an important role for PPARγ signaling in the regulation of keratinocyte immune responses.

2. Materials and methods

2.1. Keratinocyte culture

Primary normal human keratinocytes were isolated from surgically discarded neonatal skin samples. This study was conducted according to principles of the Declaration of Helsinki, and all procedures that involved human subjects received prior approval from the ethics committee at the Ehime University School of Medicine. Written consent was provided by patient guardians before experiments were initiated. Normal human keratinocytes were cultured in MCDB153 medium as described previously [14].

2.2. Adenovirus vector construction and infection

The pcDNA3 expression vectors expressing flag-tagged wild-type (wt) PPARγ and flag-tagged L468A/E471A PPARγ (dn-PPARγ) were gifts from Professor K. Chatterjee (University of Cambridge, UK). The double-mutant form of PPARγ shows impaired transcriptional activity, silences basal transcription, and is a potent dominant-negative inhibitor of wild-type PPARγ activity [16]. An adenovirus vector (Ax) containing wt-PPARγ or dn-PPARγ was generated and transfected into keratinocytes as described previously [13]. Ax1W was used as the control vector to control for effects of Ax itself.

2.3. RNA preparation and real-time RT-PCR

Total RNA from cultured cells was isolated using Isogen (Nippon Gene, Tokyo, Japan). Real-time RT-PCR was performed and analyzed in an ABI PRISM 7700 sequence detector (Applied Biosystems, Branchburg, NJ). The primers and probes used for hBD-3 (forward: 5′-TCAGCTGACTTCCAAAGGA-3′, reverse: 5′-TCAGCTGACTTCCAAAGGA-3′, probe: 5′-AACAGATCGGCAAGTGCTCGACGC-3′) and cathelicidin (forward: 5′-CACAGCAGTCACCAGAGGATTG-3′, reverse: 5′-GGCCTGGTTGAGGGTCACT-3′, probe: 5′-GGCCTGGTTG AGGGTCACT-3′) were selected using the Primer Express software (Applied Biosystems). The primers and probe for GAPDH were obtained from Applied Biosystems. RNA analysis was carried out using the TaqMan RT-PCR Master Mix Reagent Kit (Applied Biosystems), following the suggested protocol. The level of target gene expression was normalized to GAPDH and is reported as the change relative to control.

2.4. Protein preparation and Western blot analysis

Keratinocytes were harvested at specific times after treatment and whole cell lysates were extracted. Twenty micrograms of protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Analyses were performed using a Vistra ECF Kit (Amersham Biosciences, Arlington Heights, IL), and membranes were then scanned using a FluorolImager (Molecular Dynamics, Sunnyvale, CA). The following antibodies were used for Western blotting: goat anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-cathelicidin (Innovagen, Lund, Sweden), mouse anti-hBD-3 (Alpha Diagnostics, San Antonio, TX), rabbit anti-VDR, mouse anti-PPARγ and rabbit anti-Fra1 (Santa Cruz Biotechnology), as well as rabbit antibodies against c-Fos, p38, phospho-p38, phospho-ERK, and phospho-JNK (Cell Signaling Technology, Beverly, MA). The nuclear lysates used for Western blotting were extracted as described previously [17].

2.5. Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSAs)

Nuclear proteins were isolated, and the EMSA was performed as described previously [13] using a Light Shift® Chemiluminescent EMSA Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. The oligonucleotide probe sets (biotin-labeled and unlabeled probes) specific for PPARγ [13], VDRE and AP-1 [14] were obtained from Panomics (Redwood City, CA). Protein–DNA complexes were separated and transferred to Biodyne® B nylon membranes (Pierce). The biotin-labeled molecules in the membranes were detected using a Chemiluminescent Nucleic Acid Detection Module (Pierce) and exposed to X-ray film [13].

2.6. Chemicals

$1,25D_3$ was a generous gift from Teijin Pharmaceutical Co. Ltd. (Tokyo, Japan). Different concentrations of $1,25D_3$ or an equal volume of EtOH (vehicle) were added to cultures. SB203580, PD98059, and SP600125 were purchased from Calbiochem-Novabiochem International Co. (San Diego, CA) and dissolved in DMSO at 2, 30, and 20 mM, respectively, as stock solutions, and used at final concentrations of 1, 30 and 10 μM, respectively.

2.7. Statistical analysis

At least three independent experiments were performed, with similar results. One representative experiment is shown in each figure. The relative mRNA expression was expressed as the mean ± SD ($n > 3$). Statistical significance was determined using Student's paired *t*-tests. Differences were considered statistically significant at $p < 0.05$ and indicated as $^*p < 0.05$ in the figures.

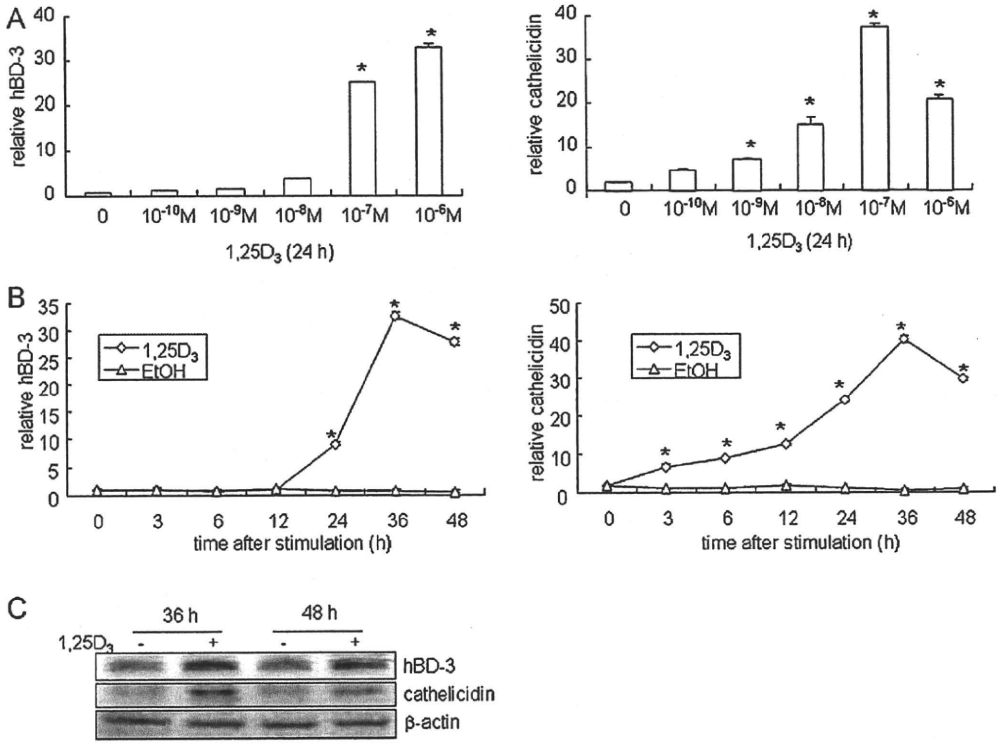

Fig. 1. $1,25D_3$ stimulates hBD-3 and cathelicidin expression. (A) Subconfluent keratinocytes were incubated with different concentrations of $1,25D_3$ for 24 h. Total RNA was collected, and real-time RT-PCR was performed to detect mRNA levels of hBD-3 and cathelicidin. (B) Keratinocytes were treated with 10^{-7} M $1,25D_3$ and harvested after the indicated times. Total RNA was analyzed by real-time RT-PCR. (C) Keratinocytes were incubated with 10^{-7} M $1,25D_3$ for 36 and 48 h, and the total extracts were subjected to Western blotting with antibodies against hBD-3, cathelicidin, and β-actin. The data represent at least three independent experiments. (*$p < 0.05$).

3. Results

3.1. Induction of antimicrobial peptides in human keratinocytes by $1,25D_3$

We first investigated AMP expression in $1,25D_3$-treated human keratinocytes. Increasing concentrations of $1,25D_3$ produced a dose-dependent upregulation of hBD-3 and cathelicidin mRNA after 24 h of treatment; significant effects were observed at concentrations greater than 10^{-7} M (Fig. 1A). Stimulation of keratinocytes with 10^{-7} M $1,25D_3$ resulted in robust induction of both hBD-3 and cathelicidin mRNA in a time-dependent manner, $1,25D_3$ produced an approximately 30-fold increase in hBD-3 mRNA expression and a more than 40-fold increase in cathelicidin mRNA expression (Fig. 1B). Additionally, cathelicidin mRNA was significantly elevated beginning 3 h, whereas hBD-3 mRNA was significantly increased beginning 12 h after treatment (Fig. 1B). Similar to what was observed with mRNA expression, $1,25D_3$ significantly upregulated hBD-3 and cathelicidin protein expression (Fig. 1C). However, $1,25D_3$ did not significantly affect hBD-1 and hBD-2 expression (data not shown). The significant induction of cathelicidin in keratinocytes by $1,25D_3$ is consistent with several previous reports [6,8]. We did not observe the rapid induction of hBD-2 by $1,25D_3$, which conflicts with the data of Wang et al. [6], but is consistent with another report [9]. Notably, our data provide the first evidence for significant upregulation of hBD-3 by $1,25D_3$ in keratinocytes.

3.2. PPARγ signal contributes to the induction of hBD-3 and cathelicidin by $1,25D_3$

We next investigated if PPARγ signaling contributes to innate immunity in keratinocytes. Keratinocytes were transfected with a vector expressing wt-PPARγ, which resulted in abundant PPARγ protein expression (Fig. 2A) and stimulated PPARγ DNA binding activity as detected by EMSA analysis (Fig. 2B). Genetically enhanced PPARγ signaling produced a 7-fold increase in hBD-3 mRNA levels and a 5-fold increase in cathelicidin mRNA (Fig. 2C). Moreover, transfection with Ax-wt-PPARγ induced the phosphorylation of p38 (Fig. 2D), and increased AP-1 DNA binding activity in keratinocytes (Fig. 2E). Pretreatment with p38 inhibitor, SB203580, not only eliminated the wt-PPARγ-induced hBD-3 expression but also significantly reduced the mRNA induction of cathelicidin (Fig. 2F). These data suggest that PPARγ signal regulates AMP expression through activating p38 pathway in human keratinocytes.

Furthermore, the priming increase of PPARγ expression strongly upregulated the $1,25D_3$-induced hBD-3 expression and sustained the $1,25D_3$-mediated induction of cathelicidin (Fig. 3A). Next, we investigated if PPARγ activity is essential for $1,25D_3$-induced AMP expression in keratinocytes. To examine the effects of PPARγ signaling on $1,25D_3$ function, we transfected keratinocytes with dn-PPARγ, which specifically blocks PPARγ signaling [16]. Transfection with dn-PPARγ reduced $1,25D_3$-induced cathelicidin mRNA expression by about 50%, and almost completely blocked the increases in hBD-3 mRNA (Fig. 3B). The dn-PPARγ also suppressed the 1,25D3-induced increases in hBD-3 and cathelicidin protein expression (Fig. 3C). These data indicate a potential role of PPARγ signaling in 1,25D3-stimulated innate immunity.

3.3. Transfection with dn-PPARγ does not affect binding of the DR-3 element

As the classical DR-3-type VDRE binding motif located in the cathelicidin promoter region [7] is responsible for $1,25D_3$-

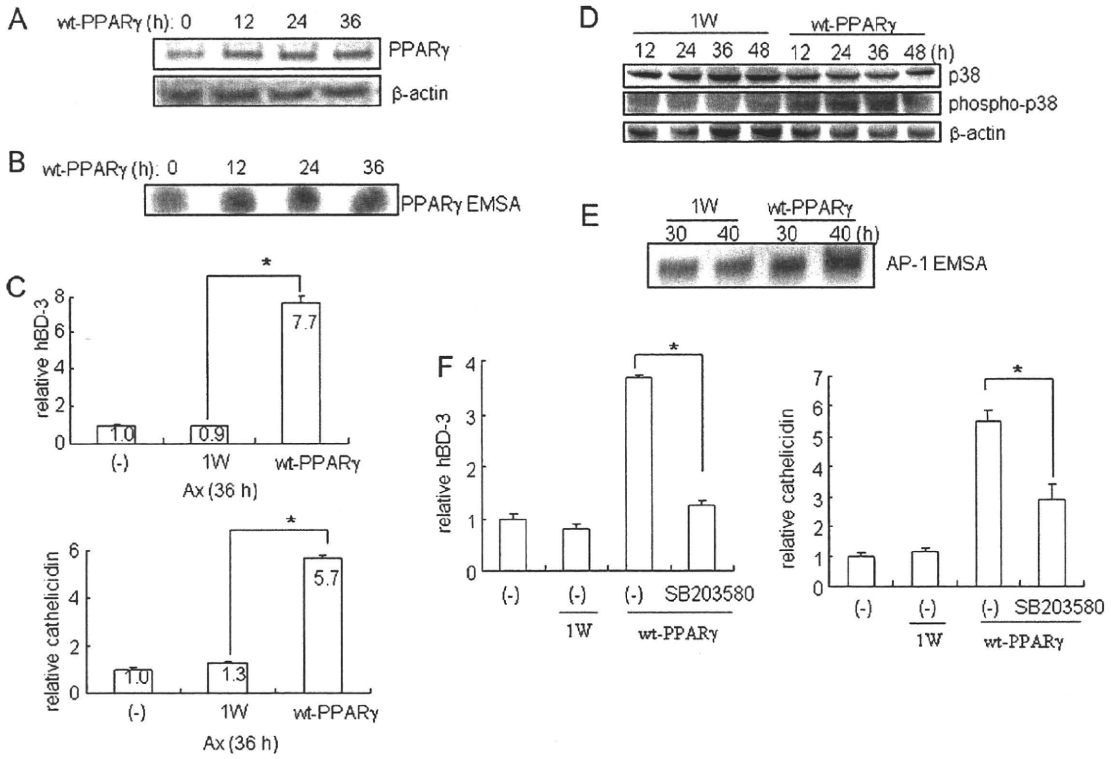

Fig. 2. wt-PPARγ induces the expression of hBD-3 and cathelicidin via activating p38 pathway. Keratinocytes were transfected with Ax-wt-PPARγ [multiplicity of infection (MOI) = 10] for 0, 12, 24, and 36 h. And cells were harvested for detection of PPARγ protein by Western blot analysis (A); and nuclear proteins were collected and incubated with biotin-labeled PPARγ probe, and EMSA was performed (B). (C) Keratinocytes were transfected with Ax-wt-PPARγ for 36 h. Total RNA was analyzed by real-time RT-PCR. Keratinocytes were transfected with Ax1W or Ax-wt-PPARγ for the indicated times, the total protein levels of p38 and phospho-p38 (D) and AP-1 DNA binding activity (E) were evaluated by western blotting and EMSA. (F) Keratinocytes were transfected with Ax-wt-PPARγ for 40 h in the presence or absence of 1 μM SB203580, the mRNA levels of hBD-3 and cathelicidin were detected using real-time RT-PCR. The data represent at least three independent experiments. (*$p < 0.05$).

mediated cathelicidin promoter activation and peptide expression in keratinocytes [6], we investigated whether PPARγ regulates cathelicidin transcription via facilitation of $1,25D_3$-induced protein binding to VDRE. Confirming a previous report [18], $1,25D_3$ rapidly induced VDR expression in keratinocytes (Fig. 4A), and stimulated the protein binding to the DR-3 element (Fig. 4B). But dn-PPARγ did not affect $1,25D_3$-induced VDR expression (Fig. 4A) and VDRE transactivation (Fig. 4B). Preincubation with unlabeled DNA blocked that protein binding, indicating that the probe used was specific (Fig. 4B). These data indicate that the VDRE in the cathelicidin promoter region is not involved in the regulation of cathelicidin induction by PPARγ in keratinocytes.

3.4. AP-1 and p38 activity contribute to PPARγ-mediated regulation of AMP induction by $1,25D_3$

As the AP-1 transcriptional binding motif has been detected in the promoter region of hBD-3 [15,19], we investigated if AP-1 activity is required for $1,25D_3$-mediated hBD-3 induction in keratinocytes. Three distinct MAPK signal pathways have been identified in mammalian cells: p38, ERK, and JNK, all of which contribute to AP-1 transcriptional activity by regulating the expression and phosphorylation of AP-1 subunits [20]. Treatment of keratinocytes with $1,25D_3$ resulted in the rapid and sustained phosphorylation of p38, ERK, and JNK (Fig. 5A), and increased AP-1 DNA-binding activity (Fig. 5D) [14]. Pretreatment with SB203580, PD98059, or SP600125, specific inhibitors of p38, ERK, and JNK, respectively, all strongly reduced hBD-3 mRNA induction in $1,25D_3$-treated keratinocytes (Fig. 5B). Despite the absence of an AP-1-binding motif in the promoter region of cathelicidin, SB203580 partially suppressed $1,25D_3$-mediated cathelicidin mRNA expression, while PD98059 and SP600125 presented no significant effect (Fig. 5B). No cytotoxic effects were produced by treatment with MAPK inhibitors. These data suggest that all three MAPK signaling pathways are critical for the transcription of hBD-3; whereas only the p38 pathway plays a role in the induction of cathelicidin mRNA in $1,25D_3$-treated keratinocytes.We next tested if PPARγ regulates $1,25D_3$-induced MAPK activation and AP-1 transactivation in keratinocytes. Transfection with dn-PPARγ significantly inhibited $1,25D_3$-induced p38 phosphorylation (Fig. 5C), but had no effect on ERK or JNK phosphorylation (data not shown). However, $1,25D_3$-induced AP-1 DNA-binding was almost completely blocked by dn-PPARγ (Fig. 5D). Moreover, dn-PPARγ suppressed the inducible total and nuclear expression of Fra1 and c-Fos (Fig. 5E and F), both of which participate in AP-1 formation and contribute to $1,25D_3$-induced AP-1 transactivation [21]. These data suggest that PPARγ regulates $1,25D_3$-induced AMPs production via p38 and AP-1 in human keratinocytes.

4. Discussion

The epidermal keratinocytes differentiate to form a physical barrier consisting of a multilayered epidermis and develop a chemical defense system based on the production of various AMPs [1,2]. We show here that $1,25D_3$ significantly induces hBD-3 and cathelicidin expression, but has no effect on hBD-1 and hBD-2 expression in cultured keratinocytes. $1,25D_3$ activates p38, ERK and JNK signaling pathways, increases c-Fos and Fra-1 expression

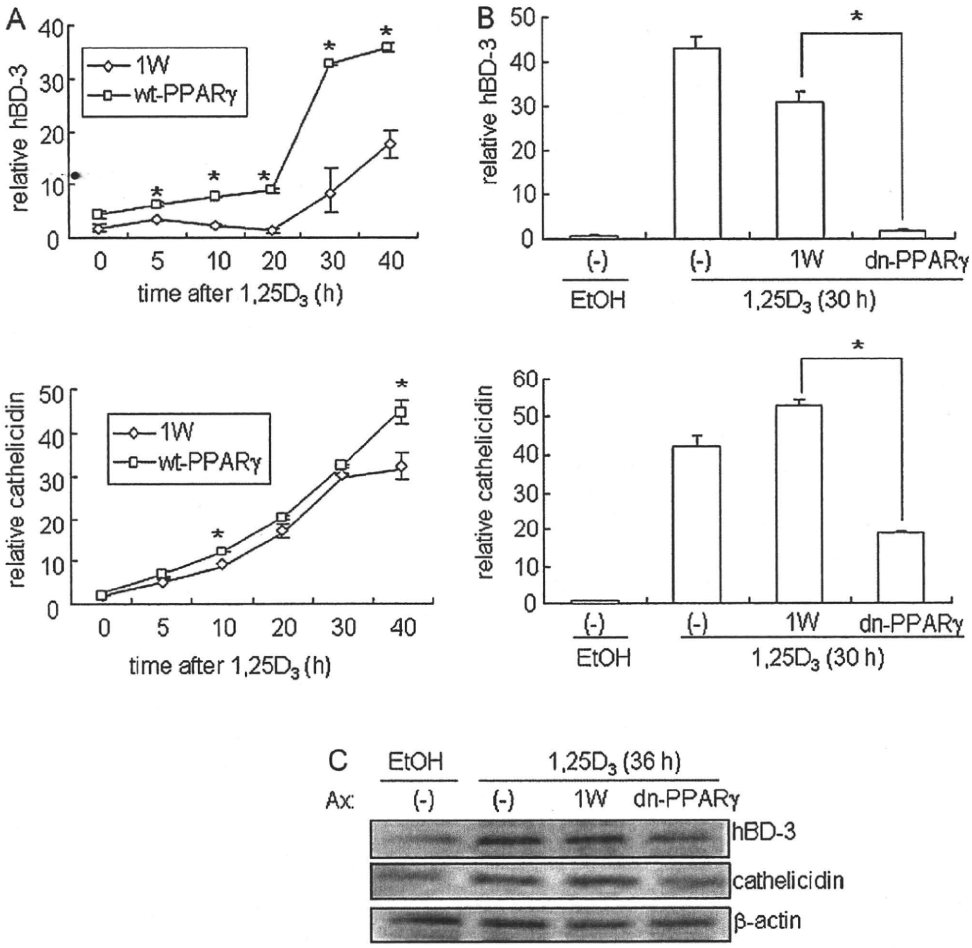

Fig. 3. dn-PPARγ interferes with the induction of hBD-3 and cathelicidin by $1,25D_3$. (A) Keratinocytes were transfected with Ax-wt-PPARγ for 12 h, then treated with $1,25D_3$ for the indicated times, the mRNA levels of hBD-3 and cathelicidin were detected using real-time RT-PCR. (B) Keratinocytes were transfected with Ax1W or Ax-dn-PPARγ (MOI = 10) for 24 h and then treated with $1,25D_3$. Total RNA was collected after 30 h, and the expression levels of hBD-3 and cathelicidin mRNA were detected using real-time RT-PCR. (C) Keratinocytes were transfected with Ax1W or Ax-dn-PPARγ and then incubated with $1,25D_3$ for 36 h, the protein levels of hBD-3 and cathelicidin were evaluated by Western blotting. The data represent at least three independent experiments. ($^*p < 0.05$).

and stimulates AP-1 transactivation in keratinocytes, which in turn induces hBD-3 transcription (Fig. 5). With respect to cathelicidin induction by $1,25D_3$, not only VDRE DNA-binding but also p38 activity is required (Fig. 5). Notably, our data provide the first evidence for the function of PPARγ in keratinocyte innate immunity. We show that PPARγ regulates AP-1 transactivation and p38 phosphorylation, which not only contributes to involucrin expression [14] but is also involved in the increased expression of hBD-3 and cathelicidin in $1,25D_3$-treated keratinocytes (Fig. 6). These data suggest a vital role for PPARγ in regulating the effects of $1,25D_3$ on keratinocyte differentiation and cell immunity.

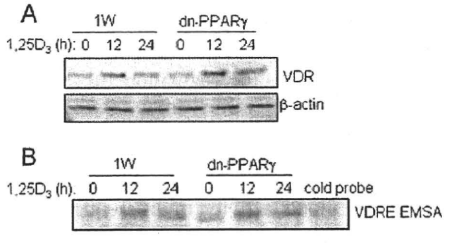

Fig. 4. dn-PPARγ has no effect on $1,25D_3$-mediated VDR expression and VDRE transactivation. Keratinocytes were transfected with Ax1W or Ax-dn-PPARγ then treated with $1,25D_3$ for the indicated times. VDR protein expression was detected by Western blot (A); and a biotin-labeled VDRE probe was incubated with the nuclear proteins, EMSA was performed (cold probe lane: an unlabeled probe was added before incubation with the biotin-labeled VDRE probe) (B). The data represent at least three independent experiments.

The multiple signaling pathways are involved in the regulation of hBDs. hBD-2 expression is induced by NF-κB, AP-1, MAPK, and PKC, whereas the hBD-3 gene promoter has no discernible NF-κB binding elements but does contain transcriptional binding motifs for AP-1, IFN-γ response elements, and NF-IL-6 response elements [3]. We show here that all three AP-1 activating MAPK signaling pathways are necessary for $1,25D_3$-mediated hBD-3 induction, indicating a central role of AP-1 DNA binding in $1,25D_3$-mediated hBD-3 transcription in keratinocytes. We also demonstrate that PPARγ contributes to AP-1 transactivation by regulating $1,25D_3$-induced c-Fos and Fra1 expression, by which PPARγ signaling regulates hBD-3 expression in keratiocytes. Fos and Jun proteins participate in AP-1 formation, with various AP-1 homodimers or heterodimers carrying out cell-specific regulation of specific genes. Incubation of keratinocytes with $1,25D_3$ increased the expression of only c-Fos and Fra1, indicating that $1,25D_3$ activates specific AP-1 subtypes, which in turn may result in selective activation of AP-1 responsive genes depending on the composition of the AP-1 dimers

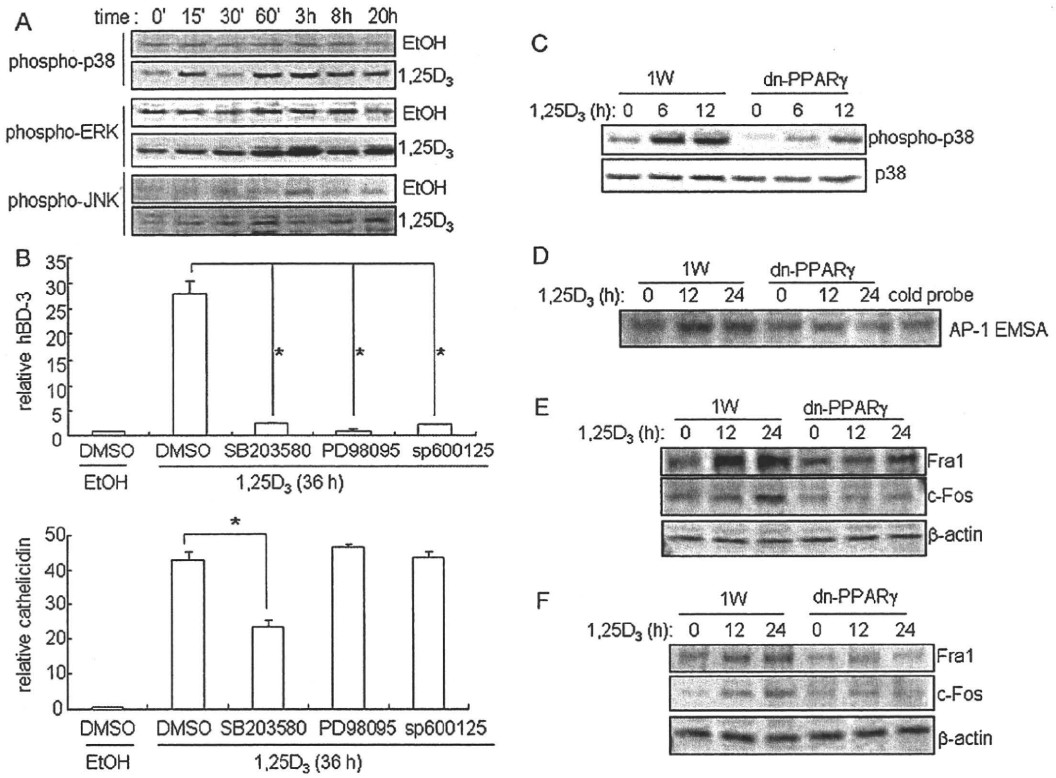

Fig. 5. Upregulation of AP-1 transactivation and p38 activity by $1,25D_3$ is inhibited by dn-PPARγ. (A) Keratinocytes were incubated with $1,25D_3$ for the indicated times, total lysates were collected, and Western blot analysis was performed to assess phopho-p38, phospho-ERK, and phospho-JNK protein levels. (B) Keratinocytes were preincubated with 1 μM SB203580, 30 μM PD98059, or 10 μM SP600125 for 1 h before $1,25D_3$ treatment. Total RNA was collected after 36 h, and the levels of hBD-3 and cathelicidin mRNA were measured using real-time PCR. Keratinocytes were infected with Ax-dn-PPARγ or Ax1W before the addition of $1,25D_3$. Cells were harvested after 6 h and 12 h, and p38 and phospho-p38 levels were evaluated by immunoblotting (C); and nuclear extracts were collected after 12 and 24 h, a biotin-labeled AP-1 probe was incubated with the nuclear proteins, and EMSA was performed (cold probe lane: an unlabeled probe was added before incubation with the biotin-labeled AP-1 probe) (D). Keratinocytes were infected with Ax-dn-PPARγ or Ax1W then treated with $1,25D_3$ for 12 and 24 h, after which the total cell lysates (E) and the nuclear extracts (F) were subjected to Western blotting to evaluate Fra1 and c-Fos protein levels. The data represent at least three independent experiments. ($^*p < 0.05$).

and promoter context. This feature may account for why $1,25D_3$ induces hBD-3 expression but not hBD-2 expression, even though AP-1 has been shown to regulate hBD-2 transcription [3].

Keratinocyte differentiation often contributes to hBD-3 expression [15,22]. Keratinocyte differentiation together with hBD-3 upregulation was induced not only by $1,25D_3$, as shown here, but also by other differentiation-inducing agents such as high calcium and retinoic acid (data not shown). These data suggest that keratinocyte differentiation, which activates AP-1, drives hBD-3 expression. We found that PPARγ signaling promotes the expression of both involucrin [14] and hBD-3, which is probably due to the ability of PPARγ to regulate keratinocyte differentiation and induce AP-1 transactivation.

Despite the absence of AP-1 motif on the promoter region of cathelicidin, the MEK–ERK pathway has been shown to be required for cathelicidin induction not only in butyrate-treated colon epithelial cells but also in $1,25D_3$-treated keratinocytes [9]. However, we found that p38 but not ERK phosphorylation contributed to cathelicidin induction by $1,25D_3$ in keratinocytes. This discrepancy may be attributable to the different culture conditions used. Although cell differentiation is not sufficient to induce cathelicidin expression [9], $1,25D_3$ and ASK1-mediated keratinocyte differentiation [15] regulate cathelicidin expression via the p38 pathway. Additionally, activation of PPARγ signaling, another differentiation-inducing event, contributes to cathelicidin expression through the regulation of p38 activity. Therefore, cell differentiation, activates p38 activity and induces cathelicidin expression at least in keratinocytes.

The expression of cathelicidin can be influenced by trans-acting enhancer element as well as by changes in transcript stability, and control may occur transcriptionally or posttranscriptionally, depending on cellular conditions. $1,25D_3$ induces cathelicidin expression in most $1,25D_3$-senstive human cells, including primary human keratinocytes, neutrophils, and monocytes [23], but not in colon epithelial cells, despite the expression of VDR in these cells [9]. $1,25D_3$ activates the cathelicidin promoter in colon epithelial cells but has no effect on mRNA and peptide expression. In contrast, despite the inability of butyrate to enhance cathelicidin promoter activity, butyrate elevates cathelicidin mRNA expression through a posttranscriptional regulation [9]. PPARγ activation most likely strengthens cathelicidin transcript stability by regulating p38 activity [24], thereby increasing cathelicidin mRNA expression, which needs further study.

Upon binding with VD3, VDR also recruits different coactivators to initiate transcriptional activity. In differentiated keratinocytes, recruitment of steroid receptor coactivators (SRCs) occurs in a cyclical manner and is required for optimal gene regulation [25]. SRC family members, such as SRC3, form complexes with VDR to recruit a number of histone acetyltransferases (HATs), which can increase HAT activity on chromatin and facilitate gene transcription [26,27]. SRC3 is strongly expressed in differentiated keratinocytes and aids in $1,25D_3$-mediated cathelicidin transcription

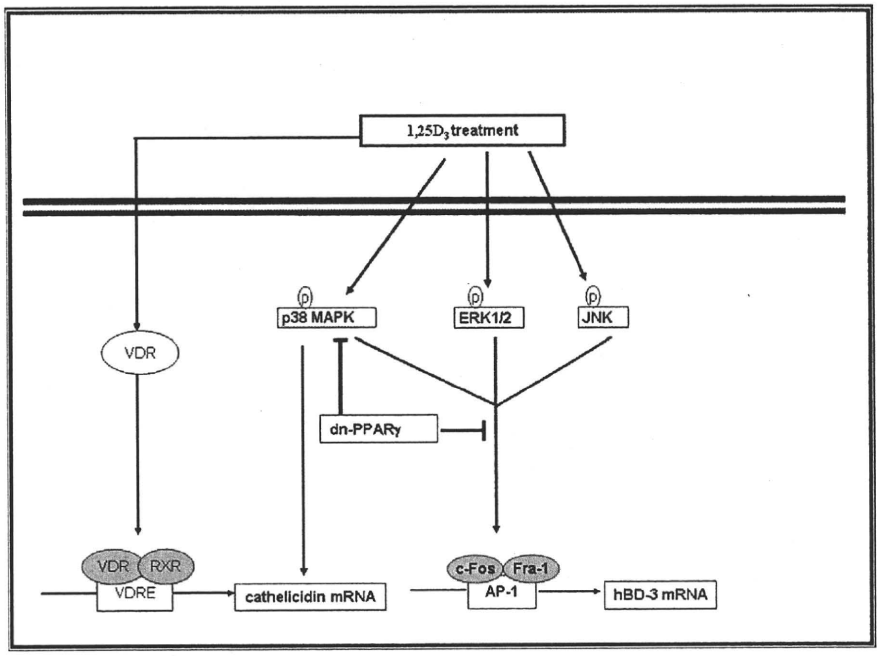

Fig. 6. A schematic model of a possible mechanism for the regulation of PPARγ on $1,25D_3$-mediated innate immune response. Treatment of keratinocytes with $1,25D_3$ activates p38, ERK and JNK MAP kinases and induces AP-1 transactivation, which in turn increases hBD-3 production. Upon the activation of VDRE, cathelicidin transcription is induced. The activation of p38 by $1,25D_3$ also contributes to the expression of cathelicidin. $1,25D_3$ stimulates PPARγ expression and signal activation, which is involved in $1,25D_3$-mediated hBD-3 and cathelicidin production by regulating AP-1 and p38 activity.

[25]. In addition to VDR, SRC family members also interact with other nuclear receptors such as RXR, LXR, and PPAR, to facilitate epidermal differentiation and cutaneous physiology [26,28]. SRC3 may be involved in the effects of PPARγ on cathelicidin expression, although this possibility requires further study.

AMPs have distinct regulatory systems, which supports their wide-ranging functional abilities. These peptides not only play an important role in the innate immune response, but also have inflammatory modulating and wound-healing capabilities [1–3,29]. $1,25D_3$ exerts multiple effects on AMP expression in keratinocytes, it not only regulates neutrophil gelatinase-associated lipocalin [6], but also induces cathelicidin and hBD-3 expression. Moreover, the effects of $1,25D_3$ on AMP expression are not limited to the VDR, as $1,25D_3$ also increases AMP expression via AP-1 and p38 pathway. Although PPARγ is best known as transcriptional regulator of lipid and glucose metabolism, evidence has also accumulated for its importance in skin homeostasis [10,12]. Here we provide the first evidence PPARγ activation facilitates the induction of hBD-3 and cathelicidin in keratinocytes following stimulation with $1,25D_3$ by regulating AP-1 and p38 activity, suggesting a potential role of PPARγ signaling in skin innate immunity.

Acknowledgments

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女性や，濃い胸毛やひげに悩む男性も多く，医学脱毛として電気脱毛術，レーザー脱毛が行われている．

a. 電気脱毛術

皮膚に接する部分が絶縁されている「特殊絶縁針」を毛根に刺入し通電することで，毛根・毛乳頭を破壊する脱毛法である．

b. レーザー脱毛術

毛根（毛包）周囲のメラニンにレーザー光線が選択的に吸収され，その熱の放散により毛根が破壊される脱毛法である．今や脱毛術の主流となっている．

●まとめ

今や時代は，メスを用いる手術からメスを用いない手術へ，ダウンタイム*1の長い手術からダウンタイムのほとんどない施術へと移行してきている．今後は，新しい手技・医療材料・医療機器・レーザーをはじめ，人工培養細胞・器官なども開発されていくことだろう．

F. 遺伝相談

●遺伝カウンセリング（遺伝相談）とは

遺伝カウンセリングの定義としてもっとも広く使われているのは米国人類遺伝学会（1975年）により提案された，「遺伝カウンセリングとは，ある家系の遺伝疾患の発症や発症のリスクに関連した人間の問題を扱うコミュニケーションの過程である．」という定義である．すなわち，患者や家族が疾患の経過，治療法，遺伝様式を理解し，特定の血縁者に再発するリスクを正しく評価したうえで再発のリスクに対応するための適切な方策を選択できるように援助することである．

*1 ダウンタイム：病床に臥している期間の意味から転じて，腫れ，出血，疼痛や，ガーゼによる被覆などによって，人前に出られない，あるいは仕事や学校に行けない期間をいうようになった．ここ1～2年の傾向として，ダウンタイムの長い手術による大きな効果を求めるよりは，治療効果は少なくてもダウンタイムがほとんどない治療を望む患者が増えている．

●遺伝カウンセリングの基本理念

遺伝カウンセリングの基本理念として以下のことに留意してカウンセリングにあたるべきである．とくに最近の遺伝子解析技術の進歩に伴い，治療法のない遺伝疾患の発症前診断や出生前診断を含むケースを扱うこともまれではなく，カウンセリングを行う際には表1に示した遺伝医学に関連した種々の倫理指針を遵守して行うべきである．

①一方的な医学的情報の伝達ではなく，相互方向のコミュニケーションプロセスである
②クライアント自身の意思がもっとも重要である
③正確かつ十分な情報の提供および得られた情報の完全な開示
④精神的・心理的援助の必要性
⑤守秘義務
⑥生命倫理の尊重

●遺伝カウンセリングの手順

遺伝カウンセリングには以下のステップがある．

①遺伝医学的に正しい病名をつける
②詳細な家系図，家系構成員の臨床症状に関する情報を集める．遺伝疾患の診断やその遺伝予後を判定するためには，詳細な家系図の作成が不可欠である．従来，家系図の書き方に世界共通のルールがなかったため，現在米国人類遺伝学会で標準化を提唱している．
③遺伝的危険率を推定する．
再発率の推定：メンデル遺伝病や遺伝性の染色体構造異常では，理論的に分離の法則に従う分離比が算定できる．これを理論的再発率という．しかしながらヒトでは分離比を乱す

表1　倫理指針

① 3省合同（文部科学省，厚生労働省，経済産業省）「ヒトゲノム・遺伝子解析研究に関する倫理指針」
② 遺伝医学関連10学会「遺伝学的検査に関するガイドライン」
③ 厚生労働省「医療・介護関係事業者における個人情報の適切な取り扱いのためのガイドライン」
④ 日本衛生検査所協会「ヒト遺伝子検査受託に関する倫理指針」

さまざまな要因により理論どおりにはならないことがある．このような場合には経験的再発率が用いられる．経験的再発率は多数の同一疾患家系の解析から得られたものである．各遺伝病の再発率などについては，成書を参照されたい．

④危険率がある程度高い場合，出生前診断，保因者診断，発症前診断などそれを回避する方法があるかどうかを示す．またそれらの検査を希望する場合には，検査のメリットとデメリットを説明したうえで，受けられる所を紹介する．

⑤上記の事柄をクライアントの文化的社会的背景，理解度を考慮しつつ正確に伝えて，意思決定の援助をする．さらにすべてのステップにおいてクライアントに対して精神的心理的サポートを行い，必要に応じて継続的にフォローアップする．

●遺伝カウンセリングを行う際に注意すべき事柄

遺伝カウンセリングはカウンセリングの担当者とクライアントの人間性の対峙であり，お互いの信頼関係を良好に築きつつ進めることが大切である．原則としてクライアントは問題を抱える個人あるいは夫婦であり，どちらかの責任という発想が生まれ心理的葛藤が起こりやすくなる．とくに，おのおのの親族が同席する場合には十分な注意が必要である．

一般に遺伝病は特殊な存在で，特殊な人だけが罹患すると思われがちであるが，誰でもが遺伝病になりうるのだということを，根拠をもって示すと心理的安定が得られやすい．

遺伝子診断希望者の増加にしたがって理解が不十分な方も増加しているため，遺伝子診断は目的をはっきりさせてから行うべきである．

遺伝子カウンセリング終了時には，今後の継続対応を保証することも重要である．

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CASE REPORT

Clinical effect of tocoretinate on lichen and macular amyloidosis

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ABSTRACT

Lichen amyloidosis and macular amyloidosis are commonly therapy-resistant. Tocoretinate is a hybrid compound of retinoic acid and tocopherol that is commonly used for the treatment of skin ulcers. Although beneficial effect of oral retinoic acid on lichen amyloidosis is reported, tocoretinate has not been reported to be useful for the treatment of lichen amyloidosis or macular amyloidosis. We evaluated the effects of topical tocoretinate on lichen amyloidosis and macular amyloidosis lesions. Tocoretinate was topically applied daily to the lesions and clinical improvement and histological changes were evaluated. The outcome was very good for four, good for two, moderate for two and poor for two of 10 treated patients. Epidermal hypertrophy was reduced and expression of involucrin, keratin 1 and keratin 10 was decreased by tocoretinate treatment, suggesting the normalization of epidermal differentiation. Amyloid deposits remained histologically detectable, even in clinically responsive patients. Together, topical application of tocoretinate reduced the clinical symptoms of lichen amyloidosis and macular amyloidosis, and normalized disturbed epidermal differentiation.

Key words: lichen amyloidosis, macular amyloidosis, retinoic acid, tocoretinate.

INTRODUCTION

Lichen amyloidosis (LA) and macular amyloidosis (MA) are primary localized cutaneous amyloidosis characterized by chronic hyperkeratotic hyperpigmented itchy papules and macules.[1,2] Histologically, amyloid deposits which are thought to be composed of degenerated keratin peptides are observed in the papillary dermis.[3] LA and MA commonly co-occur with other skin disorders such as atopic dermatitis (AD).[4]

Although topical corticosteroid is an effective treatment for milder cases of LA and MA, treatment of the majority of cases is unsatisfactory. Alternative treatments include topical dimethylsulfoxide (DMSO),[5] tacrolimus,[6] calcipotriol,[7] oral acitretin,[2,8] cyclosporine,[4] cyclophosphamide,[9] cepharanthine,[10] phototherapy (ultraviolet B, psoralen and ultraviolet A therapy),[8] laser therapy,[11,12] surgical therapy[13] and hydrocolloid dressings.[14] Herein, we evaluate the effects of topical tocoretinate, a synthetic esterified compound of tocopherol and retinoic acid, on the LA and MA lesions of 10 patients.

METHODS

Patients

Ten patients (seven men and three women) diagnosed with LA or MA by biopsy were included in this study (Table 1). Written informed consents were taken from every patient. Amyloid deposits were confirmed by thioflavin T staining. The mean age was 43 years (range 23–66), and the mean disease duration was 7.5 years (range 3–30). Nine patients had received previous treatment with topical corticosteroid. Five patients also had atopic dermatitis, and one

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Table 1. Characteristic features and outcome of lichen amyloidosis and macular amyloidosis patients treated with tocoretinate

Patient	Sex	Age (years)	Disease duration (year)	Type	Localization	Complication	Previous treatment	Improvement (month)	Outcome
1	M	28	3	LA and MA	Forearm, chest, upper back	AD	Topical steroid	2	Very good
2	M	35	30	LA	Forearm	AD	Topical steroid	1	Very good
3	M	47	15	LA	Upper arm	–	Topical steroid	1	Very good
4	M	66	3	MA	Upper back	–	Topical steroid	1	Very good
5	F	23	2	MA	Leg, upper back	AD	Topical steroid	1	Good
6	M	60	10	LA	Upper arm	–	Topical steroid	4	Good
7	F	36	3	LA	Leg	AD	–	3	Moderate
8	F	52	3	LA	Leg	–	Topical steroid	4	Moderate
9	M	33	3	LA and MA	Upper back	AD	Topical steroid	–	Poor
10	M	50	3	LA	Leg	RA	Topical steroid	–	Poor

AD, atopic dermatitis; LA, lichen amyloidosis; MA, macular amyloidosis; RA, rheumatoid arthritis.

had rheumatoid arthritis. Tocoretinate was topically applied to the LA or MA lesion of each patient twice a day and the outcome was evaluated every month.

Measurement of involucrin (IVL) and nerve growth factor (NGF) levels

Normal human epidermal keratinocytes (NHEK) were purchased from DS Pharma Biomedical (Osaka, Japan) and cultured in Human Keratinocyte Serum Free Medium (DS Pharma Biomedical) at 37°C with 5% CO_2. Culture dishes precoated with type 1 collagen (Asahi Techno Glass, Funabashi, Japan) were used to plate cells. Tocoretinate was provided by Kayaku (Saitama, Japan) and diluted in acetone. Tocoretinate at the concentration of 10 μmol/L or 100 nmol/L was added to the medium and the same concentration of acetone was added to the control sample. Cells were collected 6 h later. RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI, USA). The product was reverse-transcribed into first-strand complementary DNA (cDNA). Thereafter, the expressions of IVL and NGF were measured using the Power SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the mRNA levels. Sequence-specific primers were designed as follows: IVL sense 5′-tctgcctcagccttactgtg-3′ and antisense 5′-ggaggaggaacagtcttgagg-3′; NGF sense 5′-cagttttaccaaagggagcagctt -3′ and antisense 5′-caacatggacattacgctatgca-3′; and GAPDH sense 5′-tgtcatcatacttggcaggtttct-3′ and antisense 5′-catggccttccgtgttccta-3′. Real-time PCR (40 cycles of 92°C for 15 s and 60°C for 60 s) was run on an ABI 7000 Prism (Applied Biosystems).

Immunohistochemical staining

Lichen amyloidosis lesions were biopsied before and 3 months after applying tocoretinate. The sections were fixed in formalin and embedded in paraffin. After deparaffinization and rehydration, the sections were incubated in 3% H_2O_2 for 5 min to block endogenous peroxidase activity, and the rinsed sections were boiled in 10 mmol/L citrate buffer pH 6.0 for antigen retrieval. After blocking non-specific protein binding with 2% bovine serum albumin for 10 min, the sections were stained with primary antibody: anti-filaggrin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-involucrin antibody (Santa Cruz Biotechnology), anti-cytokeratin 1 antibody (Covance, Emiryville, CA, USA) anti-cytokeratin 10 antibody (Covance), anti-cytokeratin 14 antibody (Covance), or anti-protein gene product (PGP) 9.5 (DAKO Cytomation, Carpentaria, CA, USA) for 60 min. After washing with Tris-buffered saline (TBS) containing 0.05% Triton-X (TBST), samples were developed using the DAKO ChemMate Envision Kit/HRP (Dako Cytomation) for cytokeratins, involucrin, filaggrin statins or LSAB + System-AP (Dako Cytomation) for PGP 9.5 stain. Finally, they were counterstained with hematoxylin.

RESULTS

Three patients (30%) exhibited very good responses, two patients (20%) had good responses and three patients (30%) had moderate responses. Two

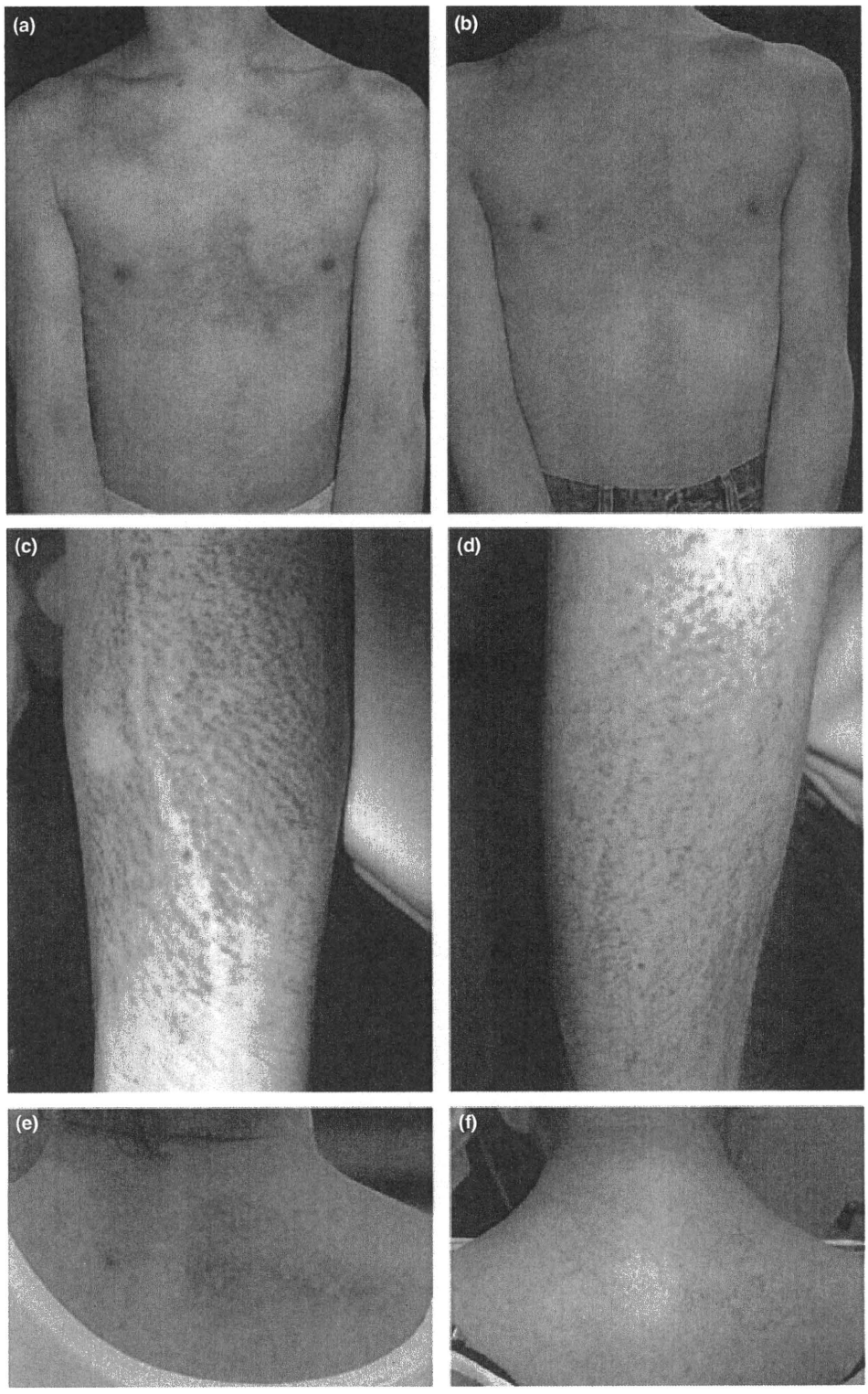

Figure 1. Clinical features of patients. Papules and macules of the chest of patient 1 before (a) and after (b) tocoretinate treatment. Papules of the forearm of patient 1 before (c) and after (d) tocoretinate treatment. Macules of the upper back of patient 4 before (e) and after (f) tocoretinate treatment.

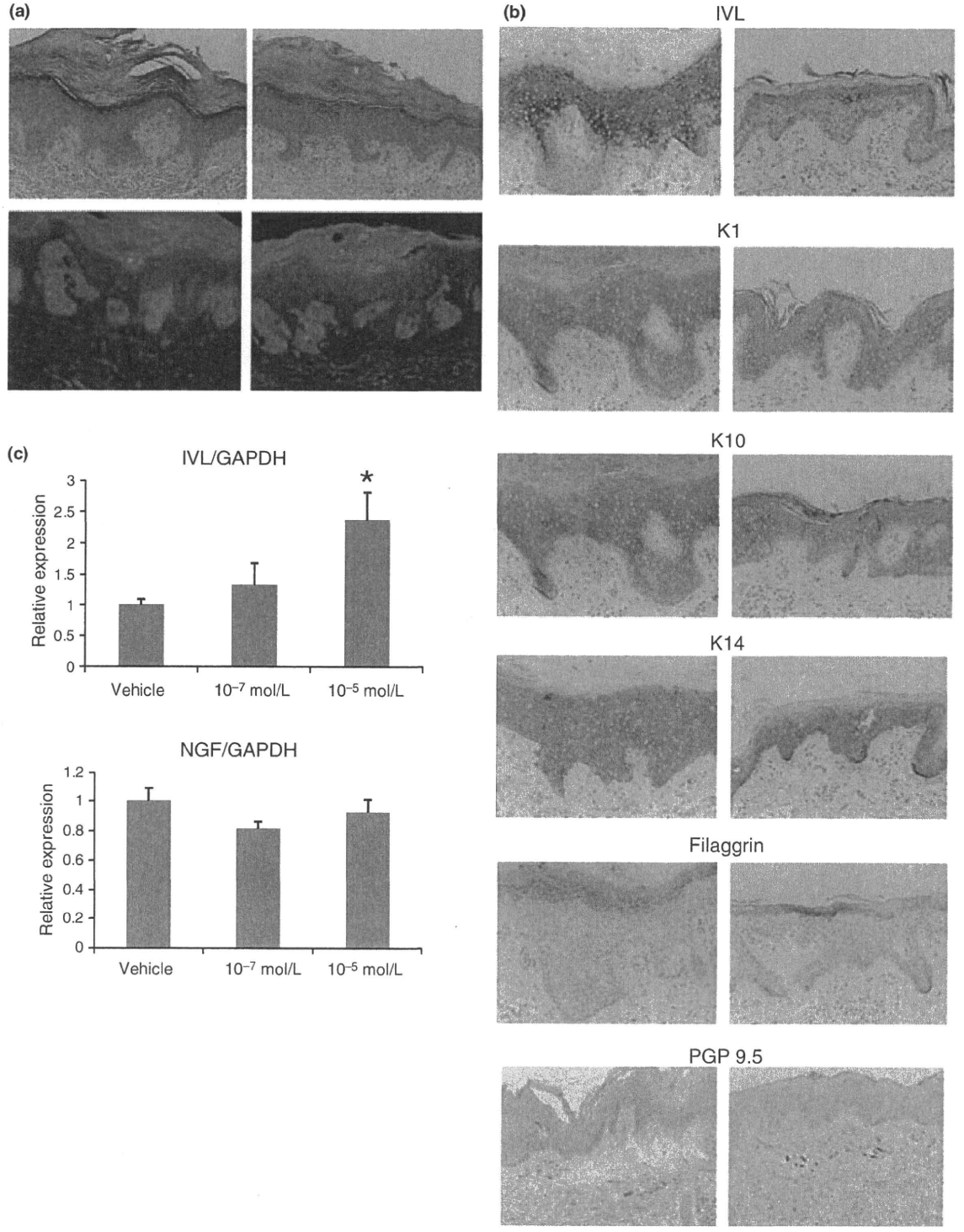

Figure 2. (a) Hematoxylin–eosin and thioflavin T staining of biopsied lichen amyloidosis lesion from patient 2 before (left) and after (right) tocoretinate treatment. (b) Immunohistochemistry of involucrin (IVL), cytokeratin 1 (K1), cytokeratin 10 (K10), cytokeratin 14 (K14), Filaggrin and protein gene product (PGP) 9.5 before (left) and after (right) tocoretinate treatment. (c) Relative expression of IVL and nerve growth factor (NGF) mRNA in normal human epidermal keratinocytes 6 h after adding tocoretinate (10^{-7} mol/L or 10^{-5} mol/L) or vehicle. Histogram shows the mean (± standard deviation). *P = 0.034 vs vehicle-treated group.

patients (20%) did not respond after 10 weeks of treatment (Table 1). The mean response duration for the responders was 2.1 months (range 1–4). Pruritus and pigmentation were reduced and papules were flattened in LA patients (Fig. 1a–d). In MA patients, pigmentation and scratch marks on the upper back were significantly reduced (Fig. 1e,f). The lesions recurred gradually after tocoretinate treatment was discontinued in four patients. Of these four patients, two patients were treated with tocoretinate again and experienced satisfactory responses.

The downward extension of the epidermis improved and the granular layer became thickened after 3 months of topical tocoretinate treatment. Although the lesions clinically improved, amyloid deposits still existed in the upper dermis (Fig. 2a). Tocoretinate treatment also normalized the disturbed epidermal differentiation in LA lesions (Fig. 2b). The width of the broadened band of involucrin staining in LA lesions was dramatically reduced in the upper spinous and granular layers by tocoretinate application. Also, the expression of the spinous layer keratins K1 and K10 was reduced by tocoretinate treatment. Expression of the suprabasal layer keratin K14, which was extended to the spinous layer in lesions, was reduced after tocoretinate treatment although still expressed in the suprabasal layer. Filaggrin expression was observed in the entire granular layer in both pre- and post-treatment samples. The amount of PGP 9.5 stain was not significantly altered between pre- and post-treatment although cutaneous innervation was more apparent in pretreated skin. The mRNA expression of the keratinocyte differentiation marker IVL was dose-dependently upregulated by tocoretinate *in vitro* treatment (Fig. 2c), suggesting that tocoretinate causes keratinocyte differentiation, rather than keratinocyte proliferation. The mRNA expression of NGF did not change significantly after tocoretinate treatment (Fig. 2c).

DISCUSSION

Tocoretinate, the synthetic compound of tocopherol and retinoic acid, has been used for the treatment of skin ulcers because it enhances fibroblast migration and proliferation.[15] It also accelerates neovascularization (manufacturer information). In addition to its beneficial effects for the treatment of skin ulcers, tocoretinate is reported to improve skin manifestations of scleroderma, morphea and hypertrophic scars.[15] Although tocoretinate has not been reported to be useful for the treatment of LA or MA, the differentiation promoting properties of tocoretinate suggests that it may act similarly to the oral amyloidosis treatment acitretin. Acitretin is reported to be effective for the treatment of LA and biphasic amyloidosis.[2] The apoptosis-inducing and phagocytosis-stimulating effects of acitretin are thought to mediate its beneficial effects for amyloidosis. We evaluated the effect of tocoretinate, the compound of tocopherol and retinoic acid in this study as tocoretinate is a widely used ointment in Japan with rare side-effects. Tocoretinate had two differentiation-promoting effects for keratinocytes which may mediate its beneficial effects for amyloidosis treatment: (i) normalized epidermal differentiation *in vivo*; and (ii) elevation of the expression of the differentiation marker involucrin *in vitro*. Even though amyloid deposits remained detectable following tocoretinate treatment, clinical improvement was evident as a result of treatment. Tocoretinate also reduced pruritus and pigmentation, suggesting that it has other actions such as increasing macrophage phagocytosis and affecting peripheral sensory nerves. We measured NGF expression of keratinocyte, although tocoretinate did not alter its expression. It is also reported that innervations of the epidermis were unexpectedly diminished in LA recently.[16] We investigated the amount of nerve fibers by staining PGP 9.5. The amount of nerve fibers in the dermoepidermal junction was not significantly altered although cutaneous innervation was more apparently observed in pretreatment skin compared with post-treatment skin. This may be because of the reduction of pruritus. Further study is needed to understand the mechanisms of the tocoretinate effects on amyloidosis. Because of the rare side-effects and fast clinical improvement within 4 months of treatment, we propose that tocoretinate is a potential treatment for LA and MA cases that do not respond to other treatments.

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Case Report

A Case of Giant Squamous Cell Carcinoma of the Buttock Possibly Arose from Syringocystadenoma and Invaded to the Rectum

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We report a rare case of giant squamous cell carcinoma of the buttock infiltrated to the rectum. The tumor may have arisen from syringocystadenoma papilliferum. Since there was no sign of metastasis, radical operation including rectal amputation was performed after successful neoadjuvant therapies. Afterwards, the patient has been alive free from disease for 15 months with no lymph node and distant organ metastasis.

1. Introduction

Squamous cell carcinoma (SCC) of the skin is one of the most common skin cancers and likely to occur on sun exposure regions. It is prevalent in men and increases with age. The incidence of SCC is increasing these days because of longevity and increased UV exposure associated with changes of lifestyle and destruction of the ozone layer. The larger and the deeper it grows, SCC is more likely to become metastatic [1].

Syringocystadenoma papilliferum (SP) is an uncommon benign lesion most frequently located on the head and neck. In a series of 100 cases of SP, one case occurred on the buttock [2]. SP is categorized as a kind of epidermal nevi which are organoid nevi arising from the pluripotential germinative cells in the basal layer of embryonic epidermis. These cells give rise not only to keratinocytes but also to skin appendages. These nevi have been often classified according to their predominant component, resulting in the terms nevus verrucosus (keratinocytes), nevus sebaceous (sebaceous glands), nevus comedonicus (hair follicles), and nevus suringocytadenosus papilliferum (or SP) (apocrine glands) [3]. Occasionally, malignant tumors develop on a preexisting SP. Many of them are basal cell carcinoma [3] less frequently SCC [4] and verrucous carcinoma [5, 6].

SCC rarely spreads from the buttock skin to the rectum although there is a report of a SCC arose from chronic perianal pyoderma that invaded around the rectum and prostate [7].

Here, we report a case of massive SCC of the buttock which may have originated from syringocystadenoma papilliferum and infiltrated deeply to the rectum.

2. Case Report

A 48-year-old male admitted to our hospital suffering from a bulky mass on his left buttock and a foul odor. The mass on his left buttock was $20 \times 10 \times 4$ cm in diameter, and its surface was cauliflower-shaped with profuse exudates and ulceration (Figure 1). The skin around the cauliflower-shaped mass colored brownish to purplish and partly had ulcers likely