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# PPARγ is an important transcription factor in 1α,25-dihydroxyvitamin D3-induced involucrin expression

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**KEYWORDS**
1α,25-Dihydroxyvitamin D3;
PPARγ;
Involucrin;
AP-1;
p38

Summary

***Background:*** 1α,25-Dihydroxyvitamin D3 ($1\alpha,25(OH)_2D_3$), the active form of vitamin D, suppresses keratinocyte proliferation, promotes keratinocyte differentiation, and induces involucrin expression. Peroxisome proliferation-activated receptors (PPARs) are ligand-activated transcription factors. It has been reported that PPARs stimulate keratinocyte differentiation and regulate the expression of differentiation molecules.
***Objective:*** Keratinocytes treated with $1\alpha,25(OH)_2D_3$ induced PPARγ, which was followed by increased involucrin expression. In this study, we investigated whether PPARγ is involved in the $1\alpha,25(OH)_2D_3$-induced involucrin expression in human keratinocytes.
***Methods:*** Subconfluent keratinocytes were treated with $10^{-7}$ M $1\alpha,25(OH)_2D_3$ for the indicated times, and PPAR and involucrin mRNA expression were determined by real-time RT-PCR. The levels of PPARs, involucrin, p38, and phospho-p38 proteins were assayed by Western blotting, and the DNA binding activities of PPARγ and AP-1 were investigated by electrophoretic mobility shift assays (EMSA). To examine the role of PPARγ in $1\alpha,25(OH)_2D_3$ responses, recombinant adenovirus carrying a dominant-negative form of PPARγ (Axdn-PPARγ) was constructed and transfected into keratinocytes. The p38 inhibitor SB203580 was added to the cultures to evaluate the involvement of p38 in involucrin expression.
***Results:*** $1\alpha,25(OH)_2D_3$ induced PPARγ expression and stimulated PPARγ activity. The introduction of dn-PPARγ inhibited the expression of involucrin mRNA and protein induced by $1\alpha,25(OH)_2D_3$, and suppressed AP-1 DNA binding activity. $1\alpha,25(OH)_2D_3$

*Abbreviations:* $1\alpha,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D3; AP-1, activator protein-1; Ax, adenovirus vector; m.o.i., multiplicity of infection; PPAR, peroxisome proliferator-activated receptor; VDR, vitamin D receptor; VDRE, vitamin D response elements.

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also triggered the phosphorylation of p38, which contributes to involucrin induction. Moreover, dn-PPARγ prevented the $1\alpha,25(OH)_2D_3$-induced phosphorylation of p38. ***Conclusions:*** These results suggest that PPARγ regulates involucrin expression by controlling the AP-1 signal and p38 activation in $1\alpha,25(OH)_2D_3$-induced keratinocyte differentiation.
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## 1. Introduction

Within the epidermis, keratinocytes migrate from the proliferative basal compartment to the upper, terminally differentiated, cornified layers of the skin, passing through a series of differentiation stages characterized by the expression of specific marker genes [1]. As involucrin is a major component of the cornified envelope, and its expression in the epidermis is tightly linked to the status of keratinocytes differentiation, involucrin is a widely used marker for keratinocyte differentiation. The activity of the involucrin promoter is complex and cell type-specific.

$1\alpha,25(OH)_2D_3$ is a hormonally active form of vitamin D, and its cellular functions are usually mediated through the nuclear hormone receptor, VDR, which binds as a heterodimer with the retinoid X receptor (RXR) to the vitamin D response element (VDRE) in the promoter region of vitamin D-responsive genes [2]. Studies have shown that $1\alpha,25(OH)_2D_3$ induces the terminal differentiation of preconfluent cultured human keratinocytes, together with elevated expression of involucrin and formation of cornified envelope [3].

PPARs are transcription factors belonging to the ligand-activated nuclear hormone receptor superfamily. On binding ligands, PPARs form heterodimers with RXR to facilitate the transcription of target genes involved in many cellular functions, including epidermal differentiation and barrier formation [4,5]. All of the PPAR superfamily members (e.g., PPARα, PPARβ/δ, and PPARγ) have been identified in keratinocytes [6], and the activation of PPARs regulate the expression of several differentiation markers [5,7]. PPARγ ligands appear to regulate cellular differentiation and cutaneous homeostasis, similar to other nuclear hormones such as glucocorticoids, retinoids, and vitamin D [8,9]. Here, we show that PPARγ signal is involved in $1\alpha,25(OH)_2D_3$-induced involucrin expression in human keratinocytes, by regulating AP-1 transactivation and p38 activity. This report suggests a vital role for PPARγ in the $1\alpha,25(OH)_2D_3$ response of cultured differentiating human keratinocytes.

## 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 the Declaration of Helsinki Principles, and all of the procedures that involved human subjects received prior approval from the Ethics Committee at the Ehime University School of Medicine, Japan. Written consent was provided by patient guardian before experiments were initiated. Normal human keratinocytes were cultured in MCDB153 medium as described previously [10].

### 2.2. Adenovirus vector construction and infection

The pcDNA3 expression vector expressing flag-tagged L468A/E471A PPARγ (dn-PPARγ) was a gift from Professor Chatterjee (University of Cambridge, UK). This double-mutant form of PPARγ exhibits impaired transcriptional activity, silences basal transcription, and is a potent dominant-negative inhibitor of wild-type PPARγ activity [11]. Adenovirus vector (Ax) containing dn-PPARγ was generated and transfected into keratinocytes as described previously [12]. Ax1W was used as the control vector to exclude the effect of Ax itself.

### 2.3. Real-time RT-PCR

Total RNA from cultured cells was isolated using Isogen (Nippon Gene, 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 GAPDH, involucrin, and the PPARs were obtained from Applied Biosystems (Norwalk, CT). The RNA analysis was carried out using a TaqMan RT-PCR Master Mix Reagent Kit (Applied Biosystems). cDNA synthesis and PCR were performed and the level of gene expression was quantified using the comparative CT method as described previously [13]. The level of target gene expression in the test samples was

normalized against GAPDH and is reported as the difference relative to GAPDH.

### 2.4. Western blotting

Keratinocytes were harvested at specific times after treatment and whole cell lysates were extracted. Twenty micrograms of protein were 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 FluorolmageR (Molecular Dynamics, Sunnyvale, CA). The following antibodies were used for Western blotting: goat anti-β-actin (Santa Cruz Biotechnology, CA), rabbit anti-involucrin (Biomedical Technologies, Stoughton, MA), rabbit anti-Fra1 (Santa Cruz Biotechnology, CA), rabbit anti-c-Fos (Cell Signaling, Beverly, MA), rabbit anti-p38 and anti-phospho-p38 (Cell Signaling, Beverly, MA), and specific antibodies against PPARα, PPARβ/δ, PPARγ (Santa Cruz Biotechnology, CA) [6,14].

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

Nuclear proteins were isolated, and the EMSA was performed as described previously [12] using a Light Shift® Chemiluminescent EMSA Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Specific PPARγ oligonucleotide probe sets (biotin-labeled and unlabeled probes) and AP-1 probe sets were obtained from Panomics (Redwood City, CA). For the supershift assays, nuclear extracts were preincubated with anti-Fra1 or anti-c-Fos antibody, or with species-matched control nonspecific IgG for 1 h at 4 °C, after which a biotin-labeled AP-1 probe was added. 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 were exposed to X-ray film [12].

### 2.6. Luciferase assay

A reporter plasmid containing the involucrin promoter and firefly luciferase (pINV-Luc) was constructed as described previously [15]. To normalize the transfection efficiency, a plasmid containing *Renilla* luciferase driven by the herpes simplex virus thymidine kinase promoter (pRL-TK; Promega, Madison, WI) was included in the assay. The reporter plasmids were introduced into keratinocytes using FuGENE6 (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. After treatment, the same number of cells was harvested with 250 μL of lysis buffer (Promega), and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) with a luminometer (Luminescencer JNR AB-2100; Atto, Japan). The relative luciferase activity was calculated by normalizing to the level of *Renilla* luciferase activity.

### 2.7. Chemicals

$1\alpha,25[OH]_2D_3$ was a generous gift from Teijin Pharmaceutical Co. Ltd. (Japan). In this study, $10^{-7}$ M $1\alpha,25[OH]_2D_3$ or same volume of EtOH (vehicle) was added into cultures. SB203580 was purchased from Calbiochem-Novabiochem International Co. (San Diego, CA) and dissolved in DMSO at 10 mM as a stock solution. One micromole SB203580 was added into cultures, and we did not detect cytotoxic effects in the keratinocytes treated with SB203580 using a cytotoxicity detection kit (Roche Diagnostics, German) (data not shown).

### 2.8. Statistical analysis

At least three independent experiments were performed, with similar results. One representative experiment is shown in each figure. The relative mRNA expression and relative luciferase activity are expressed as the mean ± S.D. ($n > 3$). 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.

## 3. Results

### 3.1. Rapid induction and activation of PPARγ by $1\alpha,25(OH)_2D_3$

First, we investigated PPAR expression in $1\alpha,25(OH)_2D_3$-treated human keratinocytes. Real-time PCR analyses revealed that PPARα mRNA remained relatively constant over time with $1\alpha,25(OH)_2D_3$ treatment. Transcription of PPARβ/δ was increased slightly after stimulation for 48 h, whereas the expression of PPARγ was induced rapidly as early as 12 h after stimulation and was upregulated by more than 10-fold at 48 h (Fig. 1a). The expression of PPAR proteins was shown in Fig. 1b, PPARα, β/δ proteins were easily detected but remained unchanged during keratinocyte differentiation, confirmed the result from high calcium-induced differentiation [6]. Robust upregulation of

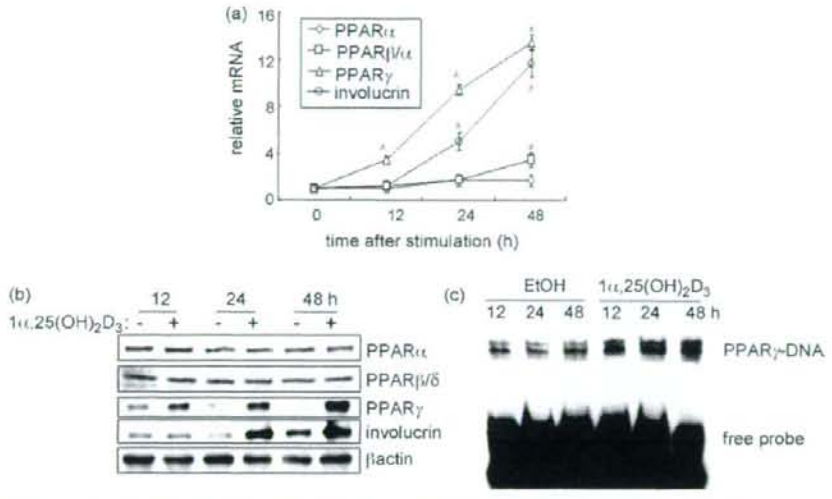


**Fig. 1** $1\alpha,25(OH)_2D_3$ stimulates rapid induction and activation of PPARγ. (a) Subconfluent keratinocytes were incubated with $1\alpha,25(OH)_2D_3$ for the indicated times (0, 12, 24, 48 h). Total RNA was collected, and real-time RT-PCR was performed. (b) Keratinocytes were treated with $1\alpha,25(OH)_2D_3$ and collected after 12, 24, or 48 h of incubation. Total proteins were analyzed by Western blotting with antibodies against involucrin, PPARs, and β-actin. (c) Keratinocytes were exposed to $1\alpha,25(OH)_2D_3$, and nuclear extracts were collected at the indicated times (12, 24, or 48 h). A biotin-labeled PPARγ probe was incubated with the nuclear proteins, and EMSA was performed. The data are representative of at least three independent experiments.

PPARγ protein was observed in $1\alpha,25(OH)_2D_3$-treated keratinocytes, beginning at 12 h and peaking at 48 h (Fig. 1b). Next, we examined whether high expression of PPARγ results in specific signal activity in $1\alpha,25(OH)_2D_3$-induced keratinocyte differentiation. We performed EMSA to examine the DNA binding activity of PPARγ using nuclear keratinocyte extracts and a PPARγ-specific probe, which could not bind to PPARα or PPARβ protein [12]. The activity was up-regulated by $1\alpha,25(OH)_2D_3$, with a significant effect observed between 12 and 48 h post-treatment (Fig. 1c). According to a previous report [6], PPARγ protein is rarely detected in undifferentiated cells; however, we detected a substantial signal in vehicle cells using Western blotting (Fig. 1b) and EMSA (Fig. 1c). The conflicting results may be attributable to different culture conditions. For example, the keratinocytes used by Westeraard et al. [6] were isolated from adult skin, and the cells were collected when they reached 30–40% confluence. In our study, primary human keratinocytes from neonatal skin were stimulated at subconfluence. Moreover, the different culture media and experimental techniques may have also affected the results. Our data suggest that PPARγ is activated in differentiating keratinocytes induced by $1\alpha,25(OH)_2D_3$.

In $1\alpha,25(OH)_2D_3$-treated human keratinocytes, involucrin was induced after the expression and activation of PPARγ (Fig. 1a and b). This could suggest a causal relationship between PPARγ and involucrin expression in $1\alpha,25(OH)_2D_3$-treated human keratinocytes.

### 3.2. Regulation of $1\alpha,25(OH)_2D_3$-induced involucrin expression by PPARγ

Recent studies have shown that PPARγ ligands stimulate differentiation and promote involucrin expression directly or synergistically with PPARβ/δ ligand in both cultured human keratinocytes [6] and murine skin following topical application [7], implying that the PPARγ signal contributes to involucrin expression.

We hypothesized that activated PPARγ is important for $1\alpha,25(OH)_2D_3$-induced involucrin expression. No synthetic or natural PPARγ antagonists have been described as completely blocking PPARγ activation; therefore, to test our hypothesis, we constructed an adenovirus vector (Ax) carrying a dominant-negative mutant of PPARγ (Axdn-PPARγ) to inactivate the PPARγ signal [11]. To investigate the role of PPARγ in the activation of the involucrin promoter, we performed a luciferase assay. The $1\alpha,25(OH)_2D_3$-induced activity of the involucrin promoter was significantly inhibited by dn-PPARγ (Fig. 2a). Moreover, dn-PPARγ also suppressed the $1\alpha,25(OH)_2D_3$-induced expression of involucrin

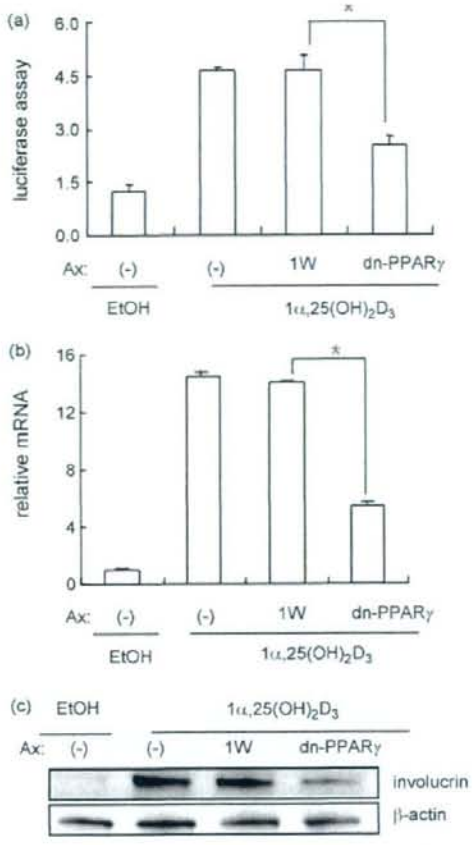


**Fig. 2** dn-PPARγ interferes with the effect of $1\alpha,25(OH)_2D_3$ on involucrin expression. (a) Keratinocytes were transfected with pINV-Luc and pRL-TK for 24 h, and then infected with Ax1W or Axdn-PPARγ for an additional 24 h. The cells were exposed to $1\alpha,25(OH)_2D_3$ for 36 h. Luciferase activity was measured using a Dual-Luciferase Reporter Assay System. The transfections were performed in triplicate. The relative luciferase activity was calculated by normalizing to the level of *Renilla* luciferase activity. (b) Keratinocytes were infected with Ax1W or Axdn-PPARγ for 24 h and then treated with $1\alpha,25(OH)_2D_3$. Total RNA was collected after 48 h, and involucrin mRNA was detected using real-time RT-PCR. (c) Keratinocytes were infected with Ax1W or Axdn-PPARγ for 24 h before the addition of $1\alpha,25(OH)_2D_3$. Cells were collected 48 h after treatment, and the level of involucrin protein was evaluated by Western blotting. The data are representative of at least three independent experiments.

mRNA (Fig. 2b) and protein (Fig. 2c) by more than 50%. Neither the control (1W) nor dn-PPARγ affected involucrin expression in vehicle cells (data not shown). These data indicate a positive role of PPARγ in involucrin expression during $1\alpha,25(OH)_2D_3$-induced keratinocyte differentiation.

### 3.3. Regulation of $1\alpha,25(OH)_2D_3$-induced AP-1 transactivation by PPARγ

According to previous reports, the AP-1 site in the involucrin promoter is essential for the induction of involucrin by $1\alpha,25(OH)_2D_3$ [16]. We hypothesized that PPARγ affects involucrin expression by controlling AP-1 transactivation. Therefore, we performed EMSA to examine the DNA binding activity of AP-1 in nuclear extracts of keratinocytes. Our results confirmed those of previous reports [17,18], treatment with $1\alpha,25(OH)_2D_3$ for 36 h significantly upregulated AP-1 DNA binding activity (Fig. 3a). In addition, dn-PPARγ essentially blocked AP-1 transactivation in $1\alpha,25(OH)_2D_3$-treated human keratinocytes (Fig. 3a). In a competition experiment, pre-incubation with an unlabeled AP-1 probe completely abolished the binding reaction, indicating that the AP-1 probe was specific (Fig. 3b). Furthermore, the addition of anti-Fra1 or c-Fos antibody resulted in a supershifted band (Fig. 3c) demonstrating the presence of these two proteins in the AP-1 DNA binding complex [18]. We also detected increased levels of Fra1 and c-Fos proteins in $1\alpha,25(OH)_2D_3$-treated human keratinocytes (Fig. 3d), and this was decreased by the expression of dn-PPARγ (Fig. 3d). The blockage of AP-1 activity in Axdn-PPARγ-infected cells might depend on the inhibition of Fra1 and c-Fos expression by dn-PPARγ (Fig. 3d). Our data suggest that PPARγ regulates involucrin expression by controlling AP-1 transactivation in $1\alpha,25(OH)_2D_3$-treated human keratinocytes.

### 3.4. PPARγ regulates $1\alpha,25(OH)_2D_3$-induced involucrin expression probably by controlling the activation of p38 MAPK

During the late stages of keratinocyte differentiation, p38 MAP kinase (MAPK) is involved in upregulating involucrin expression [19]. In addition to the phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) [18], $1\alpha,25(OH)_2D_3$ treatment resulted in a sustained increase in p38 phosphorylation (Fig. 4a). To examine whether p38 is involved in the induction of involucrin, cultures were incubated with SB203580, a specific inhibitor of p38 MAPK, before $1\alpha,25(OH)_2D_3$ treatment. The expression of involucrin in SB203580-treated vehicle cells was not different from that in untreated cells (data not shown), whereas SB203580 pretreatment of cells significantly impaired $1\alpha,25(OH)_2D_3$-induced expression of involucrin mRNA and protein (Fig. 4b and c). This suggests that p38 activity contributes to the $1\alpha,25(OH)_2D_3$-induced expression of involucrin.

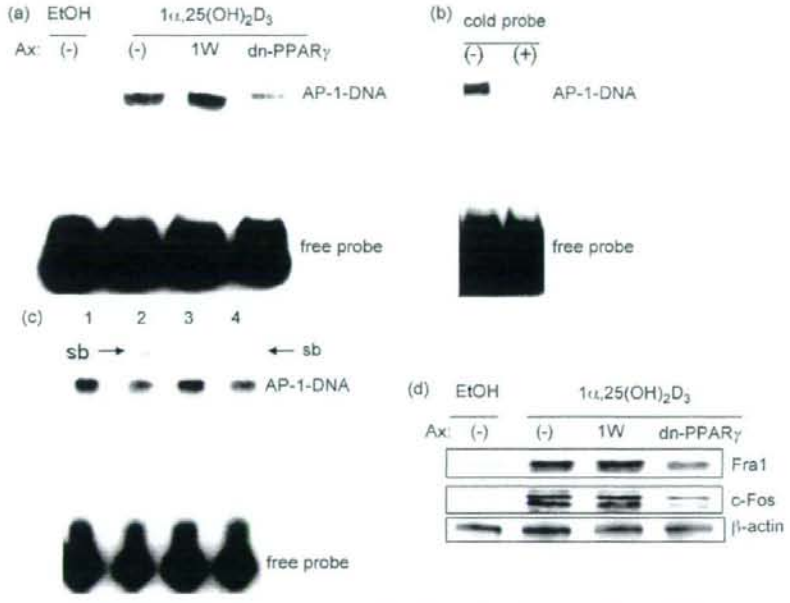


**Fig. 3** Upregulation of AP-1 transactivation by $1\alpha,25(OH)_2D_3$ is inhibited by dn-PPARγ. (a) Keratinocytes were infected with Axdn-PPARγ or Ax1W and then treated with $1\alpha,25(OH)_2D_3$. Nuclear extracts were collected 36 h after treatment. A biotin-labeled AP-1 probe was incubated with the nuclear proteins, and EMSA was performed. (b) Lane competitor (−): only a biotin-labeled AP-1 probe was incubated with the nuclear extract from $1\alpha,25(OH)_2D_3$-treated cells; lane competitor (+): pre-incubation with the AP-1 unlabeled probe. (c) Nuclear extracts from $1\alpha,25(OH)_2D_3$-treated keratinocytes were mixed with biotin-labeled AP-1 probe in the presence of antibodies. Note the supershifted band (sb) after incubation with anti-Fra1 or anti-c-Fos antibody. Lane 1: no antibody added; lanes 2–4: added rabbit anti-Fra-1 antibody, normal rabbit IgG, and rabbit anti-c-Fos antibody, respectively. (d) Keratinocytes were infected with Axdn-PPARγ or Ax1W. Total cellular extracts were collected 36 h after the addition of $1\alpha,25(OH)_2D_3$, and the Fra1 and c-Fos protein levels were detected by immunoblotting. The data are representative of at least three independent experiments.

Next, we investigated whether PPARγ regulates the activation of p38. Infection with Axdn-PPARγ nearly blocked the $1\alpha,25(OH)_2D_3$-induced phosphorylation of p38, but the total level of p38 was unaffected (Fig. 4d). This suggests that p38 activity is involved in the regulation of involucrin expression by PPARγ during $1\alpha,25(OH)_2D_3$-induced keratinocyte differentiation.

## 4. Discussion

In this study, we detected significant expression and activation of PPARγ during $1\alpha,25(OH)_2D_3$-induced keratinocyte differentiation, which is consistent with the previous reports that PPARγ expression is increased significantly during *ex vivo* and *in vitro* epidermal differentiation [6,20]. We have demonstrated increased expression and transactivation of PPARγ, but not other PPARs, in keratinocyte suspension cultures [12]. The ability of PPARγ to promote cellular differentiation and to induce the expression of differentiation-associated genes has been reported in several epithelial cells [21,22], including keratinocytes [6]. We showed that blocking PPARγ signal interfered with involucrin induction in VD3-stimulated (this study) and suspension culture-triggered keratinocyte differentiation [12], suggesting PPARγ contributes to keratinocyte differentiation by regulating the expression of involucrin.

AP-1 activity is important for both basal and inducible involucrin transcription. $1\alpha,25(OH)_2D_3$ was shown to stimulate AP-1 transactivation via the phosphatidylinositol 3-kinase/Ras/MEK/ERK/JNK signal transduction pathway [17,18]. We also detected increased phosphorylation of ERK and JNK by $1\alpha,25(OH)_2D_3$ in this study, although this is not affected by dn-PPARγ (data not shown). The activated MAPKs influence AP-1 transcriptional activity by increasing the levels of specific Jun and Fos proteins and by altering the phosphorylation of AP-1 subunits [18]. We demonstrated the involvement of PPARγ in DNA binding by AP-1 in $1\alpha,25(OH)_2D_3$-stimulated keratinocytes, which is

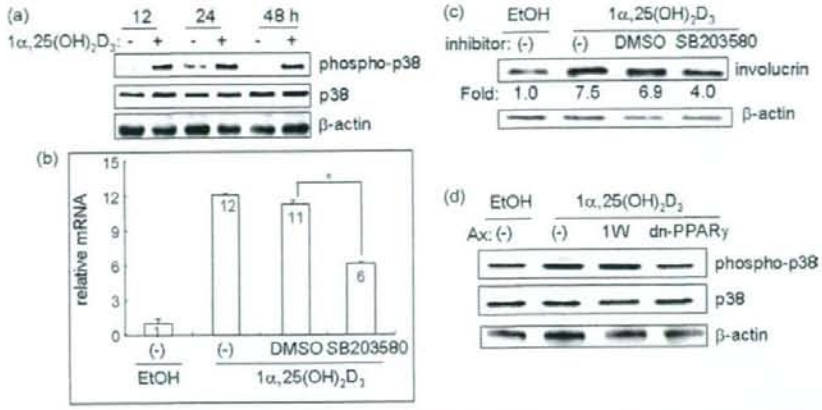


**Fig. 4** p38 is involved in the regulation of involucrin expression by PPARγ. (a) Samples were collected as described in Fig. 1b, and the levels of p38 and phospho-p38 were detected. (b) Keratinocytes were incubated with SB203580 for 1 h before $1\alpha,25(OH)_2D_3$ treatment. Total RNA was collected after 48 h, and the level of involucrin mRNA was detected using real-time PCR. (c) Keratinocytes were treated as described in (b). Total protein was collected, and involucrin expression was measured by Western blotting. The intensity of protein band was quantified using NIH Image (the control signal was defined as one unit). (d) Keratinocytes were infected with Ax1W or Axdn-PPARγ before the addition of $1\alpha,25(OH)_2D_3$. The cells were collected after 24 h, and the levels of p38 and phospho-p38 were evaluated. The data are representative of at least three independent experiments.

probably dependent on the regulation of AP-1 proteins by PPARγ. A putative VDRE containing two half sites separated by three nucleotides (DR-3) is present in the proximal promoter of involucrin and acts specifically for $1\alpha,25(OH)_2D_3$ responsiveness [16]. In human keratinocytes, the increased binding activity of the DR-3 element by $1\alpha,25(OH)_2D_3$ [16] was not affected by dn-PPARγ (data not shown). Therefore, it is reasonable that AP-1 transactivation is PPARγ-dependent and responds to the induction of involucrin by $1\alpha,25(OH)_2D_3$.

Although the involvement of p38 in $1\alpha,25(OH)_2D_3$-induced keratinocyte differentiation is not supported by a previous report [18], we found that $1\alpha,25(OH)_2D_3$ stimulation resulted in persistent activation of p38, which contributes to involucrin expression. These conflicting results on the effects of $1\alpha,25(OH)_2D_3$ on p38 activity might be attributable to differences in culture conditions. For example, Johansen [18] cultured keratinocytes isolated from adult skin and stimulated them at 50–60% confluence; we separated primary normal human keratinocytes from neonatal skin and used subconfluent cells for experiments. PPARγ agonists are able to activate p38 [23]. Blocking PPARγ activation eliminated $1\alpha,25(OH)_2D_3$-induced p38 phosphorylation, suggesting that p38 is involved in the regulation of involucrin expression by PPARγ. The effect of p38 on involucrin expression could be associated with increased binding of AP-1 and C/EBP transcription factors to the involucrin promoter [19], and post-transcriptional control of involucrin gene [13,16].

The fact that the epidermis of PPARγ-deficient mice [7] shows no detectable defect indicates that PPARγ does not play a decisive role in controlling epidermal differentiation; other transcription factors might compensate for the lack of PPARγ. Nevertheless, our data suggest that the activation of PPARγ contributes to the effect of $1\alpha,25(OH)_2D_3$ on keratinocyte differentiation. This report describes early evidence that PPARγ regulates $1\alpha,25(OH)_2D_3$-induced involucrin expression by controlling AP-1 transactivation and p38 activity in normal human keratinocytes.

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## References

[1] Fuchs E. Epidermal differentiation: the bare essentials. J Cell Biol 1990;111:2807–14.

[2] Noda M, Vogel RL, Craig AM, Prahl J, DeLuca HF, Denhardt DT. Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D3 receptor and 1,25-dihydrox-

yvitamin D3 enhancement of mouse secreted phosphoprotein 1 (SPP-1 or osteopontin) gene expression. Proc Natl Acad Sci USA 1990;87:9995–9.

[3] Smith EL, Walworth NC, Holick MF. Effect of 1 alpha, 25-dihydroxyvitamin D3 on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in serum-free conditions. J Invest Dermatol 1986; 86:709–14.

[4] Hanley K, Komuves LG, Bass NM, He SS, Jiang Y, Crumrine D, et al. Fetal epidermal differentiation and barrier development in vivo is accelerated by nuclear hormone receptor activators. J Invest Dermatol 1999;113:788–95.

[5] Kuenzli S, Saurat JH. Peroxisome proliferator-activated receptors in cutaneous biology. Br J Dermatol 2003;149: 229–36.

[6] Westergaard M, Henningsen J, Svendsen ML, Johansen C, Jensen UB, Schroder HD, et al. Modulation of keratinocyte gene expression and differentiation by PPAR-selective ligands and tetradecylthioacetic acid. J Invest Dermatol 2001;116:702–12.

[7] Mao-Qiang M, Fowler AJ, Schmuth M, Lau P, Chang S, Brown BE, et al. Peroxisome-proliferator-activated receptor (PPAR)-gamma activation stimulates keratinocyte differentiation. J Invest Dermatol 2004;123:305–12.

[8] Ellis CN, Varani J, Fisher GJ, Zeigler ME, Pershadsingh HA, Benson SC, et al. Troglitazone improves psoriasis and normalizes models of proliferative skin disease: ligands for peroxisome proliferator-activated receptor-gamma inhibit keratinocyte proliferation. Arch Dermatol 2000;136:609–16.

[9] Demerjian M, Man MQ, Choi EH, Brown BE, Crumrine D, Chang S, et al. Topical treatment with thiazolidinediones, activators of peroxisome proliferator-activated receptor-gamma, normalizes epidermal homeostasis in a murine hyperproliferative disease model. Exp Dermatol 2006;15: 154–60.

[10] Shirakata Y, Ueno H, Hanakawa Y, Kameda K, Yamasaki K, Tokumaru S, et al. TGF-beta is not involved in early phase growth inhibition of keratinocytes by 1alpha, 25(OH)2vitamin D3. J Dermatol Sci 2004;36:41–50.

[11] Gurnell M, Wentworth JM, Agostini M, Adams M, Collingwood TN, Provenzano C, et al. A dominant-negative peroxisome proliferator-activated receptor gamma (PPARgamma) mutant is a constitutive repressor and inhibits PPARgamma-mediated adipogenesis. J Biol Chem 2000;275:5754–9.

[12] Dai X, Sayama K, Shirakata Y, Hanakawa Y, Yamasaki K, Tokumaru S, et al. STAT5a/PPARgamma pathway regulates involucrin expression in keratinocyte differentiation. J Invest Dermatol 2007;1:1.

[13] Dai X, Yamasaki K, Shirakata Y, Sayama K, Hashimoto K. All-trans-retinoic acid induces interleukin-8 via the nuclear factor-kappaB and p38 mitogen-activated protein kinase pathways in normal human keratinocytes. J Invest Dermatol 2004;123:1078–85.

[14] Westergaard M, Henningsen J, Johansen C, Rasmussen S, Svendsen ML, Jensen UB, et al. Expression and localization of peroxisome proliferator-activated receptors and nuclear factor kappaB in normal and lesional psoriatic skin. J Invest Dermatol 2003;121:1104–17.

[15] Sayama K, Hanakawa Y, Shirakata Y, Yamasaki K, Sawada Y, Sun L, et al. Apoptosis signal-regulating kinase 1 (ASK1) is an intracellular inducer of keratinocyte differentiation. J Biol Chem 2001;276:999–1004.

[16] Bikle DD, Ng D, Oda Y, Hanley K, Feingold K, Xie Z. The vitamin D response element of the involucrin gene mediates its regulation by 1,25-dihydroxyvitamin D3. J Invest Dermatol 2002;119:1109–13.

[17] Johansen C, Iversen L, Ryborg A, Kragballe K. 1alpha,25-dihydroxyvitamin D3 induced differentiation of cultured human keratinocytes is accompanied by a PKC-independent regulation of AP-1 DNA binding activity. J Invest Dermatol 2000;114:1174–9.

[18] Johansen C, Kragballe K, Henningsen J, Westergaard M, Kristiansen K, Iversen L, et al. 1alpha,25-dihydroxyvitamin D3 stimulates activator protein 1 DNA-binding activity by a phosphatidylinositol 3-kinase/Ras/MEK/extracellular signal regulated kinase 1/2 and c-Jun N-terminal kinase 1-dependent increase in c-Fos, Fra1, and c-Jun expression in human keratinocytes. J Invest Dermatol 2003;120: 561–70.

[19] Eckert RL, Efimova T, Balasubramanian S, Crish JF, Bone F, Dashti S. Dashti S. p38 Mitogen-activated protein kinases on the body surface—a function for p38 delta. J Invest Dermatol 2003;120:823–8.

[20] Rivier M, Safonova I, Lebrun P, Griffiths CE, Ailhaud G, Michel S. Differential expression of peroxisome proliferator-activated receptor subtypes during the differentiation of human keratinocytes. J Invest Dermatol 1998;111:1116–21.

[21] Varley CL, Stahlschmidt J, Smith B, Stower M, Southgate J. Activation of peroxisome proliferator-activated receptor-gamma reverses squamous metaplasia and induces transitional differentiation in normal human urothelial cells. Am J Pathol 2004;164:1789–98.

[22] Fajas L, Debril MB, Auwerx J. Peroxisome proliferator-activated receptor-gamma: from adipogenesis to carcinogenesis. J Mol Endocrinol 2001;27:1–9.

[23] Teruel T, Hernandez R, Benito M, Lorenzo M. Rosiglitazone and retinoic acid induce uncoupling protein-1 (UCP-1) in a p38 mitogen-activated protein kinase-dependent manner in fetal primary brown adipocytes. J Biol Chem 2003;278:263–9. Epub 2002 October 31.

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# The NF-κB, p38 MAPK and STAT1 pathways differentially regulate the dsRNA-mediated innate immune responses of epidermal keratinocytes

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*Keywords*: chemokines, cytokine, innate immunity, IRF3, SOCS1

## Abstract

**The epidermis is the primary boundary between the body and the environment, and it serves as the first line of defense against microbial pathogens. Production of chemokines and cytokines is an important step in the initiation of innate immune responses to viral infections. Epidermal keratinocytes produce IFN-α, -β and macrophage inflammatory protein (MIP)-1α in response to double-stranded RNA (dsRNA) or viral infections. We showed that human keratinocytes produced cytokines [tumor necrosis factor (TNF)-α, IL-1β and IL-15] and chemokines [MIP-1β, RANTES and liver and activation-regulated chemokine (LARC)] in response to dsRNA, with activation of the nuclear factor κB (NF-κB), p38 mitogen-activated protein kinase (MAPK) and signal transducers and activators of transcription 1 (STAT1) pathways. To study the roles of these pathways in their production, we transfected keratinocytes with adenoviral vectors (Ax) carrying a dominant-negative form of inhibitor κB α (IκBα) (IκBαM), a dominant-negative mutant form of STAT1 (STAT1F) or suppressors of cytokine signaling 1 (SOCS1). Transfection with AxIκBαM or addition of a p38 inhibitor (SB203580) significantly decreased the dsRNA-mediated production of TNF-α, IL-1β and MIP-1α, but not of IFN-β, IL-15, MIP-1β, RANTES or LARC. Transfection with AxSTAT1F or AxSOCS1 inhibited the dsRNA-mediated production of TNF-α, IL-15, MIP-1α, MIP-1β, RANTES and LARC, but not IFN-β or IL-1β. In conclusion, the NF-κB, p38 MAPK and STAT1 pathways differentially regulate dsRNA-mediated innate immune responses in epidermal keratinocytes.**

## Introduction

The skin is the primary interface between the body and the environment and serves as the first line of defense against microbial pathogens. Epidermal keratinocytes, the main constituent of the epidermis, actively participate in innate immune responses by producing cytokines, chemokines (1–3) and anti-microbial peptides (4). In addition, human keratinocytes can be targets for viruses, such as herpes simplex virus (HSV) (5), human papillomavirus (6) and varicella-zoster virus (7). HSV- or varicella-zoster virus-infected keratinocytes are known to produce cytokines and chemokines (5, 8). Keratinocytes in virus-infected skin lesions produce macrophage inflammatory protein (MIP)-1α (7), suggesting that these cells play a role in the virus-mediated innate immune reaction of the skin. However, the regulatory mechanisms behind virus-mediated immune reaction of keratinocytes remain unclear.

Toll-like receptors (TLRs) play important roles in innate and adaptive immunity by recognizing microbial pathogens. The intracellular signals of the TLRs have been classified into myeloid differentiation primary response gene (88)-dependent and Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing IFN-beta-dependent pathways (9). TLR3 is a receptor for virus-associated double-stranded RNA (dsRNA) and activates nuclear factor κB (NF-κB), mitogen-activated protein kinases (MAPKs) and IFN regulatory factor 3 (IRF3) in an effort to control the viral infection (9, 10). In addition to TLR3, the RNA helicase retinoic acid-inducible gene (*RIG-1*) and melanoma differentiation-associated gene 5 (*MDA5*) have also been implicated in viral dsRNA recognition (11, 12). *In vitro* studies suggest that both *RIG-1* and *MDA5* detect RNA viruses and polyinosine–polycytidylic acid (polyI:C), a synthetic dsRNA analogue (11). Previously, we showed that the synthetic dsRNA, polyI:C, induced production of IFNs and MIP-1α in cultured human keratinocytes (8, 13). In airway epithelial cells, polyI:C induced the

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expression of various chemokines, including MIP-1α, MIP-1β, RANTES, 10 kDa IFN-γ-inducible protein (IP-10), IL-8 and liver and activation-regulated chemokine (LARC), as well as the expression of several cytokines, including IL-6, tumor necrosis factor (TNF)-α, IL-1β and IL-15 (14). However, production of these cytokines and chemokines in keratinocytes with dsRNA has not been studied. TNF-α and IL-1β regulate the early phase of inflammation in viral infections (15, 16), while IL-15 contributes to innate immunity by regulating the function of NK cells (17) and Langerhans cells (18). Local over-expression of IL-15 in the epidermis protects mice from cutaneous HSV infection by enhancing their anti-viral immune responses (19). Thus, it is important to study the regulatory mechanisms of the production of cytokines and chemokines in keratinocytes during viral infection to understand the defense mechanisms of the skin.

The production of most cytokines and chemokines is regulated primarily at the level of transcription, through activation of specific sets of transcription factors controlled by NF-κB, MAPKs and IRFs (13, 16). The signal transducers and activators of transcription (STAT) family plays an important role in cytokine production (20). Upon viral infection, type I IFN is rapidly induced and activates the transcription factor complex ISGF3, consisting of STAT1, STAT2 and IRF9. ISGF3 binds to *cis*-elements, termed IFN-stimulated response elements which usually reside within the promoters of IFN-inducible genes, such as *TLR3* (8) and *IRF7* (21). Some IFN-inducible genes can also be activated by IRF3 in virus-infected cells, where it forms a complex with CBP/p300 co-activators to bind to IRSE sites in the promoters of IFN-inducible genes, such as *ISG15* and *IP-10* (20, 21). The transcriptional regulation of these genes is dependent on the cellular context and stimulation (22). We reported previously that blockade of STAT1 suppressed the IFN- or polyI:C-induced expression of TLR3 and IRF7 in keratinocytes (13). However, details of the polyI:C-mediated production of other chemokines and cytokines in epidermal keratinocytes and the regulatory mechanisms involved remain unclear. Here, we report that dsRNA-induced production of chemokines and cytokines in epidermal keratinocytes was differentially regulated by NF-κB, p38 MAPK and STAT1 pathways.

## Materials and methods

### *Reagents*

PolyI:C (Amersham Biosciences Corp., Piscataway, NJ, USA) was dissolved in deionized distilled water at a concentration of 2 mg $ml^{-1}$ and stored at −70°C. SB203580 (Calbiochem–Novabiochem International Co., San Diego, CA, USA) was dissolved in dimethyl sulfoxide at a concentration of 2 mM and stored at −20°C. Anti-IRF3 antibody, anti-β-actin, anti-RIG-I and anti-MDA5 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). mAb to TLR3 was obtained from Alexis Biochemicals (San Diego, CA, USA). The antibodies for inhibitor κB α (IκBα), phospho-IκBα, p38, phospho-p38, extracellular signal-regulated kinase (ERK), phospho-ERK, c-Jun N-terminal kinase (JNK), phospho-JNK, phospho-STAT1, phospho-STAT2 and phospho-STAT3 were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).

### *Cell culture and polyI:C stimulation*

Normal human keratinocytes were cultured in MCDB153 medium, supplemented with insulin (5 μg $ml^{-1}$), hydrocortisone ($5 \times 10^{-7}$ M), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine hypothalamic extract (50 μg $ml^{-1}$) and $Ca^{2+}$ (0.03 mM), as described previously (23). Cells that had been passaged four times were used in the experiments, and subconfluent keratinocyte cultures were treated with 100 ng $ml^{-1}$ of polyI:C for a predetermined period.

This study was performed according to the principles set forth in the Declaration of Helsinki. All procedures involving human subjects received prior approval from the ethical committee of Ehime University School of Medicine. All subjects provided written informed consent to participation in the study.

### *Adenovirus construction and transfection*

AxIκBαM, AxSOCS1 and AxSTAT1F were prepared as described previously (13). Cultured normal human keratinocytes were transfected with AxIκBαM, AxSOCS1 or AxSTAT1F and then stimulated as described previously (13). AxLacZ was used as a control.

### *Immunofluorescence microscopy*

Keratinocytes on chamber slides were fixed for 5 min in methanol:acetone (1:1) and washed with PBS. The cells were then treated with anti-IRF3 antibody overnight at 4°C. After washing with PBS, the cells were incubated with fluorescein-labeled goat anti-rabbit IgG for 30 min at 37°C and then washed four times. The stained cells were visualized at a magnification of ×40 under a confocal microscope (LSM 510; Carl Zeiss, Jena, Germany).

### *Reverse Transcription–PCR*

Total RNA from the cultured cells was isolated at the indicated time points using Isogen (Nippon Gene, Tokyo, Japan). Reverse transcription (RT)–PCR was performed using RT-PCR High Plus (Toyobo, Osaka, Japan), according to the manufacturer's protocol. The primer pairs used are listed in Table 1. The products were visualized on 2% agarose gels containing ethidium bromide and were then sequenced to confirm the accuracy of amplification.

### *ELISA*

Culture supernatants were collected after treatment with polyI:C and stored at −70°C until use. ELISA kits for TNF-α and MIP-1α were purchased from Endogen (Auburn, MA, USA). ELISAs were performed according to the manufacturer's protocol. The optical density at 450 nm was measured with an Immuno Mini NJ-2300 microplate reader (Nalgene Nunc International K.K., Tokyo, Japan). All assays were performed in triplicate.

### *Protein preparation and western blotting*

The cells were harvested by transferring them into an extraction buffer containing 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM s–HCl (pH 7.4) and protease inhibitors. Equal amounts of protein were separated by

**Table 1.** The primer pairs used for RT-PCR

| Primer name | Sequence: (5'-3') |
|---|---|
| GAPDH upper | 5'-ACCACAGTCCATGCCATCAC-3' |
| GAPDH lower | 5'-TCCACCACCCTGTTGCTGTA-3' |
| IFN-β upper | 5'-CACGACAGCTCTTTCCATGA-3' |
| IFN-β lower | 5'-AGCCAGTGCTCGATGAATCT-3' |
| TNF-α upper | 5'-ACAAGCCTGTAGCCCATGTT-3' |
| TNF-α lower | 5'-AAAGTAGACCTGCCCAGACT-3' |
| IL-1β upper | 5'-AGACAAATTGCATGGTGAAGTCAGTT-3' |
| IL-1β lower | 5'-ATGGCAGAAGTACCTAAGCTCGC-3' |
| IL-15 upper | 5'-GGCTTTGAGTAATGAGAATTT-3' |
| IL-15 lower | 5'-ATCAGTTGCAATCAAGAAGTG-3' |
| MIP-1α upper | 5'-CACCATGGCTCTCTGCAAC-3' |
| MIP-1α lower | 5'-TATTTCTGGACCCACTCCTC-3' |
| MIP-1β upper | 5'-GGAATTCCCAGCCAGCTGTGGT-3' |
| MIP-1β lower | 5'-CAAGCTTGAGCAGCTCAGTTCAGTTC-3' |
| RANTES upper | 5'-GGAATTCGCCTCGCTGTCATCCTCATT-3' |
| RANTES lower | 5'-CAAGCTTGCGGTTCTTTCGGGTGACAA-3' |
| LARC upper | 5'-GGAATTCTGTACCAAGAGTTTGCTCCT-3' |
| LARC lower | 5'-CAAGCTTCGGCTATGTCCAATTCCATT-3' |

SDS–PAGE and transferred onto polyvinylidene difluoride membranes. The analysis was performed using a Vistra ECF Kit (Amersham Biosciences K.K., Tokyo, Japan) and a FluorolmagerI (Molecular Dynamics Inc., Sunnyvale, CA, USA).

*Statistical analysis*

At least three independent studies were performed and yielded similar results. The results of one representative experiment are shown in each of the figures. Quantitative data are expressed as means ± standard deviations. Statistical significance was determined using the paired Student's *t*-test. Differences were deemed statistically significant at $P < 0.05$. The levels of statistical significance are indicated in the figures as follows: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

## Results

*Polyl:C enhanced mRNA expression of cytokines and chemokines in normal human keratinocytes*

The presence of dsRNA during viral infection induces numerous inflammatory mediators in a variety of cell types (16, 22). As an initial step, we analyzed mRNA induction by polyI:C in cultured normal human keratinocytes. The levels of mRNA expression of inflammatory cytokines, including IFN-β, TNF-α, IL-1β and IL-15, and chemokines, including MIP-1α, MIP-1β, RANTES and LARC, were enhanced by polyI:C (Fig. 1a). The mRNA level of IL-18 was unchanged (data not shown).

The expression of the dsRNA detectors (24) TLR3, RIG-I and MDA5 in cultured human keratinocytes was also investigated using western blotting. As shown in Fig. 1(b), unstimulated cells (time, 0 h) expressed TLR3, but not RIG-I or MDA5. On stimulation with polyI:C, the level of TLR3 protein increased rapidly, and significant RIG-I and MDA5 expression was induced after 12 h of stimulation. The induction of RIG-I by polyI:C confirmed previous reports in human astrocytoma cells (25) and fibroblasts (26).

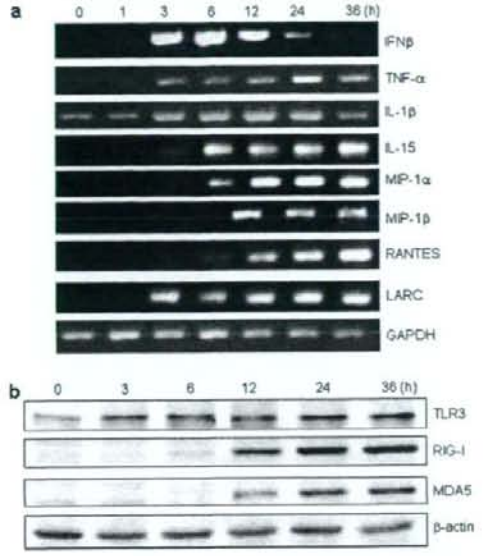


**Fig. 1.** PolyI:C enhanced mRNA expression of cytokines and chemokines in keratinocytes. (a) Keratinocytes were stimulated with polyI:C (100 ng ml$^{-1}$) for the indicated times, and RT-PCR was performed to detect the transcription of GAPDH, IFN-β, TNF-α, IL-1β, IL-15, MIP-1α, MIP-1β, RANTES and LARC. (b) Keratinocytes were stimulated with polyI:C for the indicated times, and the levels of TLR3, RIG-1 and MDA5 proteins were detected using western blotting.

*NF-κB regulated polyI:C-induced TNF-α, IL-1β and MIP-1α production*

As NF-κB regulates the production of many inflammatory cytokines (16), we examined its involvement in the polyI:C-induced production of cytokines and chemokines in keratinocytes. IκBα was rapidly phosphorylated by polyI:C (Fig. 2a), as reported previously (13). Next, the keratinocytes were transfected with an adenoviral vector (Ax) carrying a dominant-negative mutant form of IκBα (IκBαM) to block NF-κB signaling (13). Transfection with IκBαM significantly suppressed the mRNA induction of TNF-α, IL-1β and MIP-1α by polyI:C, while the levels of mRNA expression of other genes were unaffected (Fig. 2b). ELISAs for TNF-α and MIP-1α confirmed the inhibitory effect of IκBαM on their production (Fig. 2c). Thus, NF-κB signaling appeared to be involved in the induction of TNF-α, IL-1β and MIP-1α by polyI:C in keratinocytes.

*p38 MAPK regulated polyI:C-induced TNF-α, IL-1β and MIP-1α production*

The MAPK system is activated by infection with several viruses (16, 22). It has previously been shown in epithelial cells that dsRNA activated p38 MAPK, which is required for the induction of TNF-α and IL-1β (27). Considering the important role MAPK signaling plays in host defense, we examined the activation of three major MAPK subfamilies, p38, ERK1/2 and JNK, in keratinocytes upon polyI:C stimulation.

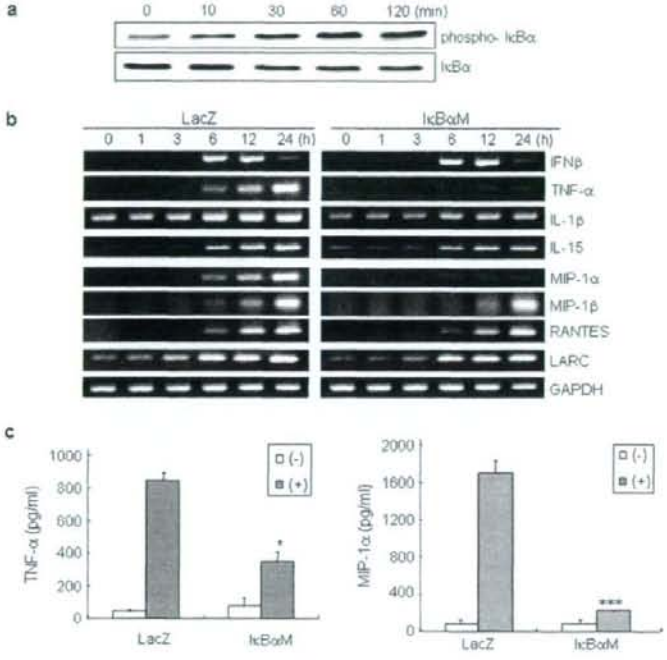


**Fig. 2.** NF-κB is involved in the polyI:C-induced expression of TNF-α, IL-1β and MIP-1α in keratinocytes. (a) Keratinocytes were treated with polyI:C for the indicated times, cell extracts were prepared and IκBα and phospho-IκBα expression were detected by western blotting. (b) Keratinocytes were infected with AxLacZ or AxIκBαM for 24 h, and the cultures were stimulated with polyI:C for the indicated times. RT-PCR was performed to determine the mRNA expression of various cytokines and chemokines. (c) Keratinocytes were infected with AxLacZ or AxIκBαM and then stimulated with polyI:C for 30 h. The supernatants were collected, and the expression of TNF-α and MIP-1α was analyzed by ELISA.

As shown in Fig. 2(a), after adding polyI:C, phospho-p38 was increased significantly, whereas the changes in phospho-ERK and phospho-JNK were not remarkable. To determine whether p38 is involved in polyI:C-induced cytokine and chemokine production in keratinocytes, p38 activity was inhibited using SB203580. Treatment with SB203580 resulted in a partial, but statistically significant, reduction in polyI:C-induced TNF-α, IL-1β and MIP-1α mRNA expression (Fig. 3B). This result was confirmed by ELISA (Fig. 3c). These observations suggest that polyI:C-induced production of TNF-α, IL-1β and MIP-1α involves p38 signaling.

*Expression of suppressors of cytokine signaling 1 suppressed the activation of STATs and reduced polyI:C-induced TNF-α, IL-15, MIP-1α, MIP-1β, RANTES and LARC production*

The JAK–STAT pathway is involved in immune regulation via IFNs (13), which are induced by viral infection and dsRNA. The suppressors of cytokine signaling (SOCS)/cytokine-inducible SH2-containing protein family, which is induced by STAT activation, negatively regulates the JAK–STAT pathway by inhibiting STAT activation (28). Previously, we showed that the STAT1–SOCS1 pathway regulates innate immunity in keratinocytes (13). In this study, we investigated whether the JAK–STAT pathway was involved in the production of cytokines and chemokines in polyI:C-treated keratinocytes. As in our previous report (13), we detected significant phosphorylation of STAT1, STAT2 and STAT3 upon polyI:C stimulation (Fig. 4a), which was blocked by SOCS1 (Fig. 4a), while the total protein levels of STATs were unchanged (data not shown). Furthermore, SOCS1 inhibited the mRNA induction of most of the genes, including the cytokines TNF-α and IL-15 and the chemokines MIP-1α, MIP-1β, RANTES and LARC (Fig. 4b). The data for TNF-α and MIP-1α were confirmed at the protein level by ELISA (Fig. 4c).

*Expression of SOCS1 blocked the polyI:C-induced nuclear translocation of IRF3*

It has been suggested that the action of SOCS is not confined to JAK/STAT signaling (29). We found that SOCS1 not only suppressed STAT activation (Fig. 4a) but also blocked the polyI:C-induced nuclear translocation of IRF3 (Fig. 4d), which is upstream of the JAK–STAT pathway during dsRNA signaling. These observations suggested that SOCS1 plays a central role in regulating the immune response in polyI:C-treated keratinocytes.

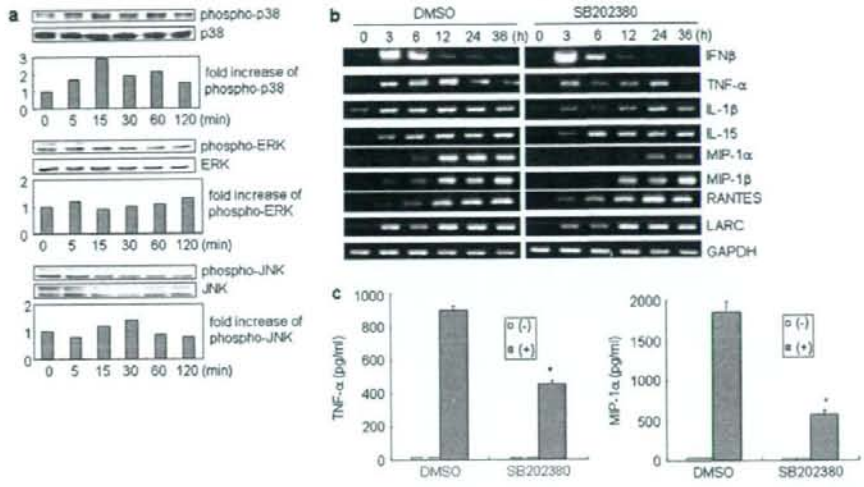


**Fig. 3.** Activation of the p38 pathway is required for polyI:C-induced TNF-α, IL-1β and MIP-1α expression in keratinocytes. (a) Keratinocytes were treated with polyI:C for the indicated times, cell extracts were prepared and the levels of p38, phospho-p38, ERK, phospho-ERK, JNK and phospho-JNK were determined by western blotting. The intensity of the protein band was quantified using NIH Image (Molecular Dynamics), and the relative increases in phospho-p38, phospho-ERK and phospho-JNK compared with the total protein are shown with the data. (b) Cultures were processed with dimethyl sulfoxide or SB202380 (20 μM) for 30 min prior to treatment with polyI:C for the indicated times. RNA was prepared, and RT-PCR was performed to detect the mRNA expression of several cytokines and chemokines. (c) Cultures were pre-treated with dimethyl sulfoxide or SB202380 for 30 min prior to stimulation with polyI:C for 30 h, and the supernatants were collected. TNF-α and MIP-1α production were examined by ELISA.

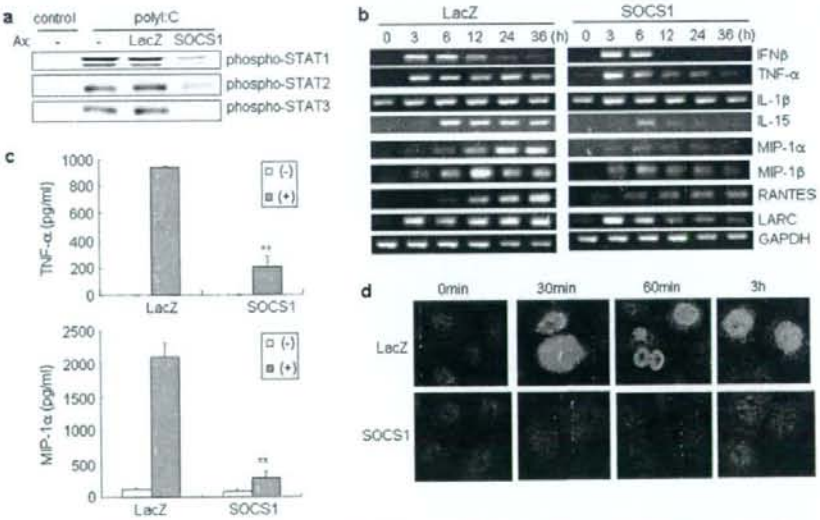


**Fig. 4.** Over-expression of SOCS1 suppressed the induction of TNF-α, IL-15, MIP-1α, MIP-1β, RANTES and LARC and blocked the nuclear translocation of IRF3 in polyI:C-treated keratinocytes. (a) Keratinocyte cultures were transfected with AxLacZ or AxSOCS1 for 24 h, stimulated with polyI:C for 4 h and the extracts were subjected to western blotting to detect phospho-STAT1, phospho-STAT2 and phospho-STAT3. (b) Keratinocytes were transfected with AxLacZ or AxSOCS1 for 24 h prior to treatment with polyI:C for the indicated time periods, and the transcription of several cytokines and chemokines was assayed by RT-PCR. (c) Keratinocyte cultures were transfected with AxLacZ or AxSOCS1 for 24 h. PolyI:C was added to the cultures, and the supernatants were collected after 30 h. ELISA was performed to evaluate the levels of TNF-α and MIP-1α. (d) Keratinocytes were transfected with AxLacZ or AxSOCS1 for 24 h prior to treatment with polyI:C for the indicated times. The cells were then fixed, and immunofluorescence was used to detect the nuclear translocation of IRF3.

*STAT1 regulated polyI:C-induced TNF-α, IL-15, MIP-1α, MIP-1β, RANTES and LARC production*

A specific role of STAT1 activation in MIP-1α production in dsRNA signaling has been demonstrated (13). We examined whether STAT1 activation was essential for polyI:C-induced cytokine and chemokine expression. An Ax carrying a dominant-negative mutant form of STAT1 (STAT1F), which specifically blocks STAT1 activation with no effect on other STATs (13), was transfected into keratinocytes. These keratinocytes were then treated with polyI:C. STAT1F regulated the mRNA expression of polyI:C-induced cytokines and chemokines in a pattern similar to SOCS1 (Fig. 5a). Moreover, STAT1F decreased the production of TNF-α and MIP-1α (Fig. 5b). As STAT1F did not influence the dsRNA-induced activation of IRF3 (data not shown), the similarities between the effects of STAT1F and those of SOCS1 on polyI:C-triggered inflammation suggest that STAT1 contributed to the response to dsRNA in keratinocytes.

## Discussion

We found that human keratinocytes produced TNF-α, IL-1β, IL-15, MIP-1β, RANTES and LARC following exposure to dsRNA, in addition to MIP-1α and IFN-β. Figure 6 summarizes the regulatory mechanisms of the polyI:C-induced production of cytokines and chemokines. Specific sets of transcription factors required for cytokine and chemokine production vary among cell types (16, 22). In airway epithelial cells, the p38 MAPK pathway alone regulates the dsRNA-induced production of TNF-α and IL-1β (27). In contrast, in keratinocytes, both p38 MAPK and NF-κB are required for the production of TNF-α and IL-1β. This may be explained by the observation that the p38 MAPK pathway is required for the efficient activation of NF-κB in response to viral infection (30) and other environmental stresses (31). In airway epithelial cells (14) and NIH 3T3 cells (32), both the IRF3 pathway and NF-κB signal are required for dsRNA-induced expression of RANTES. On the other hand, p38 MAPK regulates viral infection-induced RANTES in bronchial epithelial cells (33) and in microglia (30), but not in fibroblasts (32). In keratinocytes, STAT1, but not the NF-κB or p38 pathways, is required for RANTES production. Although all the NF-κB, p38 MAPK and STAT1 pathways regulate polyI:C-induced MIP-1α production in keratinocytes, NF-κB signaling is not essential for MIP-1α production in airway epithelial cells (14). Moreover, dsRNA-mediated LARC production is regulated by NF-κB activation in airway epithelial cells (14), but dsRNA-activated STAT1 signal is essential for LARC production in human keratinocytes. Although dsRNA elicits innate immune reactions in a variety of organs, the regulatory mechanisms to produce cytokines and chemokines differ among them. We also noted that the *IFN-β*, *TNF-α* and *LARC* genes were induced much earlier compared with other cytokines or chemokines, in response to polyI:C stimulation. In addition, inhibition of the STAT1 signal significantly suppressed the late phase, but not the early phase, of TNF-α and LARC production. This kinetic regulation of TNF-α and LARC demonstrates that different transcription factors contribute to the early and late phases of expression of these genes. In particular, the early phase of TNF-α and LARC induction might occur in response to direct dsRNA receptor-mediated TNF-α and LARC transcription, which might be activated by NF-κB and p38, although neither blocking the NF-κB signal nor inhibiting p38 resulted in a remarkable

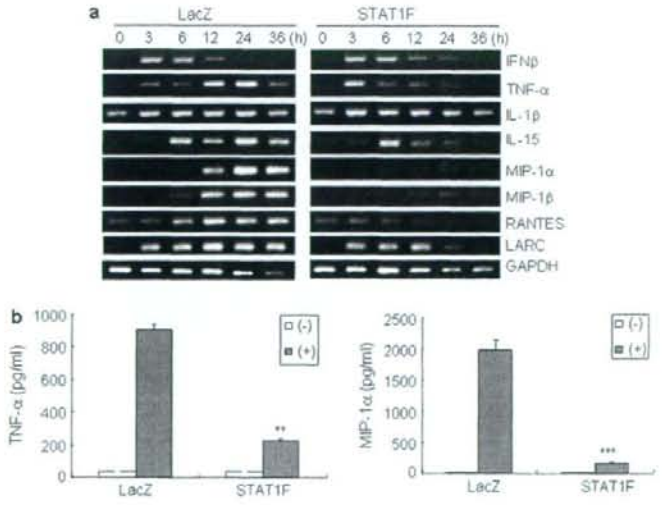


**Fig. 5.** STAT1F inhibited the induction of TNF-α, IL-15, MIP-1α, MIP-1β, RANTES and LARC by polyI:C in keratinocytes. (a) Keratinocytes were transfected with AxLacZ or AxSOCS1 for 24 h prior to treatment with polyI:C for the indicated time periods, and the transcription of several cytokines and chemokines was measured by RT-PCR. (b) Keratinocytes were infected with AxLacZ or AxSTAT1F and then stimulated with polyI:C for 30 h. The supernatants were collected, and the levels of TNF-α and MIP-1α were analyzed by ELISA.

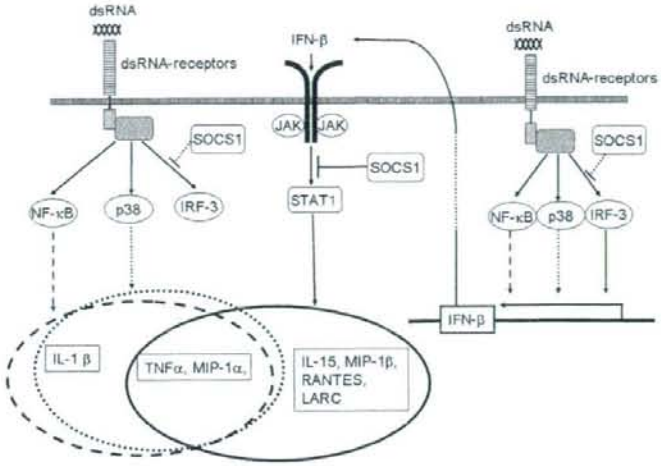


**Fig. 6.** Regulation of dsRNA-mediated cytokines and chemokines by different signaling pathways in keratinocytes. After infection with virus or treatment with dsRNA, NF-κB, p38 MAPK and IRF3 are activated, resulting in IFN-β production. The *de novo*-expressed IFN-β stimulates activation of the JAK–STAT pathway. The activation of NF-κB, p38 MAPK and JAK–STAT pathways differentially regulates dsRNA-mediated production of cytokines and chemokines.

decrease in LARC production. As opposed to early and direct TNF-α and LARC induction, the late phase of the production of these genes is indirect and is most probably mediated by dsRNA-induced IFN-β, which activates STAT1 and amplifies the dsRNA signaling (13).

*TLR3*, *RIG-I* and *MDA5* have been implicated in the recognition of dsRNA and the subsequent induction of anti-viral responses (24). In the endosome, the viral dsRNA and its mimic polyI:C are recognized by TLR3, whereas RIG-I and MDA5 have been identified as cytosomal dsRNA detectors. Moreover, RIG-I and MDA5 also activate NF-κB and IRF3 and stimulate the subsequent production of type I IFNs and pro-inflammatory cytokines when stably expressed in cells (24, 34). Despite this knowledge, the exact contributions of *TLR3*, *RIG-I* and *MDA5* to dsRNA-mediated signal transduction and cytokine production have yet to be clarified. In human astrocytoma cells, polyI:C-induced RANTES up-regulation is significantly inhibited by siRNA for *RIG-I* (25), and over-expression of RIG-I in gingival fibroblasts enhances the production of IL-1β and IL-8 induced by polyI:C (26). However, the knockdown of *RIG-I* and *MDA5* through siRNA transfection failed to inhibit polyI:C-mediated RANTES, IP-10 and IL-8 production in airway epithelial cells (35). In this study, we demonstrated that cultured normal human keratinocytes express substantial TLR3, but not MDA5 and RIG-I, suggesting that the primary reactions triggered by polyI:C are most probably mediated by TLR3, while RIG-I and MDA5, both of which are significantly induced by polyI:C, might contribute to the amplification of dsRNA signaling in keratinocytes. Further research is required to elucidate the respective functions of *TLR3*, *RIG-I* and *MDA5* in dsRNA signaling and anti-viral responses in human keratinocytes.

Our finding that SOCS1 blocked polyI:C-induced IRF3 nuclear translocation was unexpected. The mechanism of IRF3 activation by polyI:C is probably dependent on the kinases I kappa B kinase ε and TRAF-associated NF-κB activator (TANK)-binding kinase 1 (36). Although Kinjyo *et al.* (29) have suggested that SOCS1 suppressed TRAF6-dependent IKK activation when over-expressed, no studies have described the effect of SOCS in the region directly upstream of IRF3. Further research is required to clarify the molecular mechanism by which SOCS1 inhibited IRF3 activation.

Induction of type I IFNs is one of the earliest events in viral infections, in fact preceding the generation of a specific immune response (37). The promoter of the human IFN-β gene is complex, with several partially overlapping positive and negative regulatory elements (38). Three families of transcription factors, IRF3 (39), NF-κB and AP-1 (38, 40), all of which are activated in response to dsRNA or viral infection, have been shown to participate in the induction of IFNs. Activation of the IFN-β promoter requires the coordinated action of several transcription factors (41); however, not all these transcription factors may be necessary for dsRNA-mediated IFN-β induction if one of them is present in excess (42). In the current study, we found that SOCS1 over-expression blocked the activation of IRF3, but that it had no effect on IFN-β expression; furthermore, both the introduction of AxIκBαM and treatment with a p38 MAPK inhibitor failed to block IFN-β induction. It has been shown that individual *cis*-elements, in the absence of the others, can drive dsRNA-induced transcription of transfected reporter genes, indicating that each element is capable of communicating with the basal transcription machinery and with the relevant co-activators (38), and this may apply to the complex promoter of the *IFN-β* gene in polyI:C-treated keratinocytes. Compared with the regulation of the other cytokines and chemokines, the contribution of the many overlapping signaling mechanisms in *IFN-β* transcription suggests that *IFN-β* is a key element in viral infection.

Overall, our findings provide insight into the precise roles of NF-κB, p38 and STAT1 in the virus-provoked innate immune responses of epidermal keratinocytes.


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## Abbreviations

| | |
|---|---|
| Ax | adenovirus vector |
| dsRNA | double-stranded RNA |
| ERK | extracellular signal-regulated kinase |
| HSV | herpes simplex virus |
| IκB | inhibitor κB |
| IκBαM | dominant-negative mutant form of IκBα |
| IRF | IFN regulatory factor |
| JNK | c-Jun N-terminal kinase |
| LARC | liver and activation-regulated chemokine |
| MAPK | mitogen-activated protein kinase |
| MDA5 | melanoma differentiation-associated gene 5 |
| MIP | macrophage inflammatory protein |
| NF-κB | nuclear factor κB |
| poly I:C | polyinosine–polycytidylic acid |
| RIG-1 | RNA helicase retinoic acid-inducible gene |
| RT | reverse transcription |
| SOCS | suppressors of cytokine signaling |
| STAT | signal transducers and activators of transcription |
| STAT1F | dominant-negative mutant form of STAT1 |
| TLR | Toll-like receptor |
| TNF | tumor necrosis factor |

## References


1 Luger, T. A. and Schwarz, T. 1990. Evidence for an epidermal cytokine network. *J. Invest. Dermatol.* 95:100S.
2 Fujisawa, H., Kondo, S., Wang, B., Shivji, G. M. and Sauder, D. N. 1997. The expression and modulation of IFN-alpha and IFN-beta in human keratinocytes. *J. Interferon Cytokine Res.* 17:721.
3 Tohyama, M., Sayama, K., Komatsuzawa, H. *et al.* 2007. CXCL16 is a novel mediator of the innate immunity of epidermal keratinocytes. *Int. Immunol.* 19:1095.
4 Sayama, K., Komatsuzawa, H., Yamasaki, K. *et al.* 2005. New mechanisms of skin innate immunity: ASK1-mediated keratinocyte differentiation regulates the expression of beta-defensins, LL37, and TLR2. *Eur. J. Immunol.* 35:1886.
5 Mikloska, Z., Danis, V. A., Adams, S., Lloyd, A. R., Adrian, D. L. and Cunningham, A. L. 1998. *In vivo* production of cytokines and beta (C-C) chemokines in human recurrent herpes simplex lesions—do herpes simplex virus-infected keratinocytes contribute to their production? *J. Infect. Dis.* 177:827.
6 Cho, Y. S., Kang, J. W., Cho, M. *et al.* 2001. Down modulation of IL-18 expression by human papillomavirus type 16 E6 oncogene via binding to IL-18. *FEBS Lett.* 501:139.
7 Nahass, G. T., Penneys, N. S. and Leonardi, C. L. 1996. Interface dermatitis in acute varicella-zoster virus infection: demonstration of varicella-zoster virus DNA in keratinocytes by *in situ* polymerase chain reaction. *Br. J. Dermatol.* 135:150.
8 Tohyama, M., Dai, X., Sayama, K. *et al.* 2005. dsRNA-mediated innate immunity of epidermal keratinocytes. *Biochem. Biophys. Res. Commun.* 335:505.
9 Takeda, K. and Akira, S. 2005. Toll-like receptors in innate immunity. *Int. Immunol.* 17:1.
10 Matsumoto, M., Funami, K., Oshiumi, H. and Seya, T. 2004. Toll-like receptor 3: a link between toll-like receptor, interferon and viruses. *Microbiol. Immunol.* 48:147.
11 Yoneyama, M., Kikuchi, M., Natsukawa, T. *et al.* 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* 5:730.
12 Kang, D. C., Gopalkrishnan, R. V., Wu, Q., Jankowsky, E., Pyle, A. M. and Fisher, P. B. 2002. mda-5: an interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. *Proc. Natl Acad. Sci. USA* 99:637.
13 Dai, X., Sayama, K., Yamasaki, K. *et al.* 2006. SOCS1-negative feedback of STAT1 activation is a key pathway in the dsRNA-induced innate immune response of human keratinocytes. *J. Invest. Dermatol.* 126:1574.
14 Matsukura, S., Kokubu, F., Kurokawa, M. *et al.* 2006. Synthetic double-stranded RNA induces multiple genes related to inflammation through Toll-like receptor 3 depending on NF-kappaB and/or IRF-3 in airway epithelial cells. *Clin. Exp. Allergy* 36:1049.
15 Krunkosky, T. M., Fischer, B. M., Martin, L. D., Jones, N., Akley, N. J. and Adler, K. B. 2000. Effects of TNF-alpha on expression of ICAM-1 in human airway epithelial cells *in vitro*. Signaling pathways controlling surface and gene expression. *Am. J. Respir. Cell Mol. Biol.* 22:685.
16 Mogensen, T. H. and Paludan, S. R. 2001. Molecular pathways in virus-induced cytokine production. *Microbiol. Mol. Biol. Rev.* 65:131.
17 Carson, W. E., Fehniger, T. A., Haldar, S. *et al.* 1997. A potential role for interleukin-15 in the regulation of human natural killer cell survival. *J. Clin. Invest.* 99:937.
18 Mohamadzadeh, M., Berard, F., Essert, G. *et al.* 2001. Interleukin 15 skews monocyte differentiation into dendritic cells with features of Langerhans cells. *J. Exp. Med.* 194:1013.
19 Loser, K., Mehling, A., Apelt, J. *et al.* 2004. Enhanced contact hypersensitivity and antiviral immune responses *in vivo* by keratinocyte-targeted overexpression of IL-15. *Eur. J. Immunol.* 34:2022.
20 Taniguchi, T. and Takaoka, A. 2002. The interferon-alpha/beta system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. *Curr. Opin. Immunol.* 14:111.
21 Nakaya, T., Sato, M., Hata, N. *et al.* 2001. Gene induction pathways mediated by distinct IRFs during viral infection. *Biochem. Biophys. Res. Commun.* 283:1150.
22 Melchjorsen, J., Sorensen, L. N. and Paludan, S. R. 2003. Expression and function of chemokines during viral infections: from molecular mechanisms to *in vivo* function. *J. Leukoc. Biol.* 74:331.
23 Shirakata, Y., Ueno, H., Hanakawa, Y. *et al.* 2004. TGF-beta is not involved in early phase growth inhibition of keratinocytes by 1alpha,25(OH)2vitamin D3. *J. Dermatol. Sci.* 36:41.
24 Meylan, E. and Tschopp, J. 2006. Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. *Mol. Cell* 22:561.
25 Yoshida, H., Imaizumi, T., Lee, S. J. *et al.* 2007. Retinoic acid-inducible gene-I mediates RANTES/CCL5 expression in U373MG human astrocytoma cells stimulated with double-stranded RNA. *Neurosci. Res.* 58:199.
26 Kubota, K., Sakaki, H., Imaizumi, T. *et al.* 2006. Retinoic acid-inducible gene-I is induced in gingival fibroblasts by lipopolysaccharide or poly IC: possible roles in interleukin-1beta, -6 and -8 expression. *Oral Microbiol. Immunol.* 21:399.
27 Meusel, T. R. and Imani, F. 2003. Viral induction of inflammatory cytokines in human epithelial cells follows a p38 mitogen-activated protein kinase-dependent but NF-kappa B-independent pathway. *J. Immunol.* 171:3768.
28 Gadina, M., Hilton, D., Johnston, J. A. *et al.* 2001. Signaling by type I and II cytokine receptors: ten years after. *Curr. Opin. Immunol.* 13:363.

29 Kinjyo, I., Hanada, T., Inagaki-Ohara, K. *et al.* 2002. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. *Immunity* 17:583.
30 Nakamichi, K., Saiki, M., Sawada, M. *et al.* 2005. Rabies virus-induced activation of mitogen-activated protein kinase and NF-kappaB signaling pathways regulates expression of CXC and CC chemokine ligands in microglia. *J. Virol.* 79:11801.
31 Schulze-Osthoff, K., Ferrari, D., Riehemann, K. and Wesselborg, S. 1997. Regulation of NF-kappa B activation by MAP kinase cascades. *Immunobiology* 198:35.
32 Melchjorsen, J. and Paludan, S. R. 2003. Induction of RANTES/CCL5 by herpes simplex virus is regulated by nuclear factor kappa B and interferon regulatory factor 3. *J. Gen. Virol.* 84:2491.
33 Kujime, K., Hashimoto, S., Gon, Y., Shimizu, K. and Horie, T. 2000. p38 Mitogen-activated protein kinase and c-jun-NH2-terminal kinase regulate RANTES production by influenza virus-infected human bronchial epithelial cells. *J. Immunol.* 164:3222.
34 Takeuchi, O. and Akira, S. 2007. Recognition of viruses by innate immunity. *Immunol. Rev.* 220:214.
35 Matsukura, S., Kokubu, F., Kurokawa, M. *et al.* 2007. Role of RIG-I, MDA-5, and PKR on the expression of inflammatory chemokines induced by synthetic dsRNA in airway epithelial cells. *Int. Arch. Allergy Immunol.* 143:80.
36 Biron, C. A. 2001. Interferons alpha and beta as immune regulators—a new look. *Immunity* 14:661.
37 De Maeyer, E. and De Maeyer-Guignard, J. 1998. Type I interferons. *Int. Rev. Immunol.* 17:53.
38 Maniatis, T., Falvo, J. V., Kim, T. H. *et al.* 1998. Structure and function of the interferon-beta enhanceosome. *Cold Spring Harb. Symp. Quant. Biol.* 63:609.
39 Sato, M., Tanaka, N., Hata, N., Oda, E. and Taniguchi, T. 1998. Involvement of the IRF family transcription factor IRF-3 in virus-induced activation of the IFN-beta gene. *FEBS Lett.* 425:112.
40 Wathelet, M. G., Lin, C. H., Parekh, B. S., Ronco, L. V., Howley, P. M. and Maniatis, T. 1998. Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer *in vivo*. *Mol. Cell* 1:507.
41 Munshi, N., Yie, Y., Merika, M. *et al.* 1999. The IFN-beta enhancer: a paradigm for understanding activation and repression of inducible gene expression. *Cold Spring Harb. Symp. Quant. Biol.* 64:149.
42 Peters, K. L., Smith, H. L., Stark, G. R. and Sen, G. C. 2002. IRF-3-dependent, NFkappa B- and JNK-independent activation of the 561 and IFN-beta genes in response to double-stranded RNA. *Proc. Natl Acad. Sci. USA* 99:6322.

trying dietary manipulation (30%) is similar to the 40% using CAM to treat proven allergic contact dermatitis.[1] However, the majority of our patients reported that their skin failed to improve as a result of dietary manipulation. Of ongoing concern is the significant number of patients who attempt dietary manipulation without seeking appropriate expert advice.

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## References

1 Nolesen E, Munk MD, Larsen K et al. Use of complementary and alternative treatment for allergic contact dermatitis. Br J Dermatol 2007; **157**:301–5.
2 Nicolaou N, Johnston GA. The use of complementary medicine in patients referred to a contact dermatitis clinic. Contact Dermatitis 2004; **51**:30–3.
3 Johnston GA, Bilbao R, Graham-Brown RAC. The use of complementary medicine in children with atopic dermatitis in Leicester. Br J Dermatol 2003; **149**:466–71.
4 Baron ED, Barzilai D, Johnston G et al. Atopic dermatitis management: comparing the treatment patterns of dermatologists in Japan, U.S.A. and U.K. Br J Dermatol 2002; **147**:710–15.
5 Johnston GA, Bilbao R, Graham-Brown RAC. The use of dietary manipulation by parents of children with atopic dermatitis. Br J Dermatol 2004; **150**:1186–7.

Key words: allergic contact dermatitis, complementary and alternative medicine, diet

Conflicts of interest: none declared.

## Chromosomal integration of human herpesvirus 6 DNA in anticonvulsant hypersensitivity syndrome

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Sir, Anticonvulsant hypersensitivity syndrome (AHS), which is also referred to as drug rash with eosinophilia and systemic symptoms (DRESS) or drug-induced hypersensitivity syndrome (DIHS), is a multiorgan systemic reaction characterized by rashes, fever, lymphadenopathy, leucocytosis with eosinophilia and atypical lymphocytes, liver dysfunction and a close relationship to the reactivation of herpesvirus, especially human herpesvirus 6 (HHV-6), in patients on long-term anticonvulsant therapy.[1–3] AHS tends to show relatively later onset (2–8 weeks or more after commencing administration of the causative drug) than the other types of drug eruption, and HHV-6 DNA is detected in the serum at around 3–5 weeks after the onset, followed by a dramatic rise in anti-HHV-6 IgG titres.[3] We report a patient with AHS caused by carbamazepine, in whom serum HHV-6 DNA levels had persistently been extremely high and there had been chromosomal integration of the HHV-6 genome.

A 47-year-old Japanese man was admitted to our hospital on April 7th 2005, with a 12-day history of generalized erythematous rash and a fever. He had a 20-year history of schizophrenia. He had been treated with chlorpromazine hydrochloride, levomepromazine, biperiden, nitrazepam, bromazepam and clonazepam for 2 years. On January 7th 2005, oral carbamazepine had been added to his treatment. The erythematous lesions began on the 78th day of administration of carbamazepine, becoming generalized over the next 5 days. Physical examination on admission revealed a diffuse erythema with scaling over the whole body (Fig. 1). He had a high fever (38.8 °C) and bilateral cervical and inguinal lymphadenopathy. He was negative for Nikolsky's sign. Laboratory investigations revealed white blood cell count $23.5 \times 10^9$ L$^{-1}$, with 35% eosinophils and 6% atypical lymphocytes; aspartate aminotransferase 70 IU L$^{-1}$; alanine aminotransferase 73 IU L$^{-1}$; γ-glutamyltransferase 129 IU L$^{-1}$; lactate dehydrogenase 686 U L$^{-1}$; hypogammaglobulinaemia (IgG 609 mg dL$^{-1}$, IgA 34 mg dL$^{-1}$, IgM 27 mg dL$^{-1}$) and C-reactive protein 2.6 mg dL$^{-1}$. Analysis of peripheral blood lymphocyte surface markers showed 49.3% CD4+ T cells and 39.0% CD8+ T cells. Hepatitis B surface antigen, hepatitis C virus antibody, human immunodeficiency virus-1 antibody and adult T-cell leukaemia-associated antigen were all negative. Skin biopsy from the abdomen revealed hydropic and vacuolar degeneration of epidermal basal cells, lymphocytic infiltration in the epidermis and a dense upper dermal infiltrate consisting mainly of mononuclear cells. Histological examination of inguinal lymph nodes and ultrasonography of the cervical and inguinal lymph nodes bilaterally showed a benign lymphoid hyperplasia. The diagnosis of an AHS due to carbamazepine was made and carbamazepine therapy was discontinued. Because previous reports have demonstrated that cross-reactivity to multiple drugs with different structures including those used after onset of the rash can be detected in patients with DRESS/DIHS,[4] we mainly use systemic corticosteroid for treatment of these cases. Oral prednisolone 20 mg daily was given for 3 days but this was ineffective, so the dose was increased to 60 mg daily with good clinical effect. The dose was tapered over the next 21 days in line with improvement of clinical symptoms. We also gave intravenous immunoglobulin (5 g daily for 3 days, from the first day of the higher steroid dose), as recommended by other groups.[5,6] It took 6 months for complete resolution of all symptoms including eosinophilia and lymphadenopathy.

After the rash had resolved completely, we conducted patch testing with all drugs the patient had been taking before admission. A positive reaction (++) according to the International Contact Dermatitis Research Group scoring system was observed only for carbamazepine. We also performed a drug-induced lymphocyte stimulation test (DLST) to identify the causative drug. The DLST stimulation index for carbamazepine was 391% (cut-off for DLST is 180%).

Six weeks after onset, HHV-6 IgG titres increased 128-fold compared with those at admission but anti-HHV-6 IgM titres

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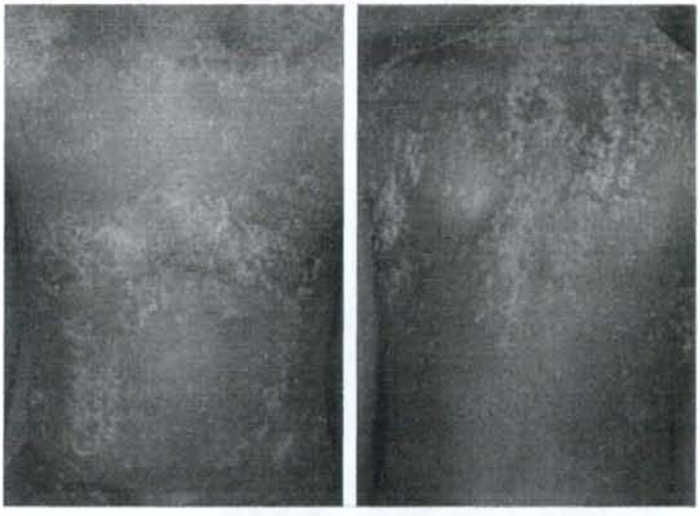

Fig 1. Clinical features on admission. Diffuse erythema with scaling on the trunk, consistent with erythroderma.

did not change during the whole course, indicating that HHV-6 reactivation had occurred in this patient. We also examined our patient's HHV-6 DNA in serum samples. In DRESS/DIHS, HHV-6 DNA has previously been detected in the serum at around 3–5 weeks after onset (and only at this point),[3] whereas in our patient HHV-6 DNA was persistently extremely high (> 10 000 copies $mL^{-1}$ serum). Our patient also showed persistently very high HHV-6 DNA levels in the whole blood.

Because the HHV-6 genome has been detected in several human lymphoproliferative disorders,[7,8] and found to be integrated into chromosomes in some cases, we examined whether our patient carried chromosomally integrated HHV-6 DNA. Fluorescent *in situ* hybridization (FISH) with the HHV-6-specific ph6Z-101 probe was performed on metaphase chromosomes from the peripheral blood as previously described.[7,8] The result of FISH analysis in this patient showed the symmetrical doublet hybridization signals seen on chromosome 1q44 (Fig. 2).

HHV-6 latency in adults is usually characterized by a very low copy number of HHV-6 genome in peripheral blood mononuclear cells.[9,10] In this respect, chromosomally integrated HHV-6 with a high copy number of HHV-6 genome should be distinct from latency after primary infection.[9] Recently, Ward *et al.* demonstrated that immunocompetent individuals who show persistently very high HHV-6 DNA levels in whole blood and serum have viral integration that has been inherited chromosomally from either of their parents.[9] Although we could not get agreement from the patient's family to look for the HHV-6 DNA in their peripheral blood, this patient's viral genome was most likely inherited chromosomally from either of his parents as described above.

This is the first report in which chromosomally integrated HHV-6 DNA is identified in an allergic disease such as AHS. Further investigation of these phenomena will improve the understanding of adverse drug reactions by providing further insight into the mechanisms underlying immune responses to viruses.

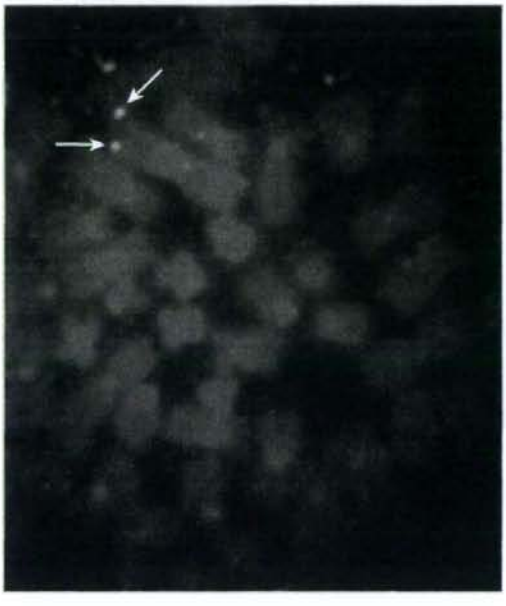

Fig 2. Fluorescent *in situ* hybridization on metaphase chromosomes from the patient. Hybridization with a human herpesvirus 6 (HHV-6)-specific probe showed HHV-6 integration with symmetrical doublet signals at homologous sites on both chromatids. Arrows indicate the hybridization signals on chromosome locus 1q44. Chromosomes were counterstained with propidium iodide. Ethical approval was sought from the Ethics Committee of Showa University School of Medicine, and the patient and his parents provided written, informed consent.

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## References

1 Bocquet H, Bagot M, Roujeau JC. Drug-induced pseudolymphoma and drug hypersensitivity syndrome (Drug Rash with Eosinophilia and Systemic Symptoms: DRESS). *Semin Cutan Med Surg* 1996; **15**:250–7.
2 Shiohara T, Iijima M, Ikezawa Z, Hashimoto K. The diagnosis of a DRESS syndrome has been sufficiently established on the basis of typical clinical features and viral reactivations. *Br J Dermatol* 2007; **156**:1083–4.
3 Seishima M, Yamanaka S, Fujisawa M *et al.* Reactivation of human herpesvirus (HHV) family members other than HHV-6 in drug-induced hypersensitivity syndrome. *Br J Dermatol* 2006; **155**:344–9.
4 Shiohara T, Kano Y. Are viral infections responsible for the development of drug-induced hypersensitivity syndrome as well as graft-versus-host diseases? *Dermatology* 2005; **210**:259–60.
5 Scheuerman O, Nofech-Moses Y, Rachmel A, Ashkenazi S. Successful treatment of antiepileptic drug hypersensitivity syndrome with intravenous immune globulin. *Pediatrics* 2001; **107**:e14.
6 Kano Y, Inaoka M, Shiohara T. Association between anticonvulsant hypersensitivity syndrome and human herpesvirus 6 reactivation and hypogammaglobulinemia. *Arch Dermatol* 2004; **140**:183–8.
7 Daibata M, Taguchi T, Sawada T *et al.* Chromosomal transmission of human herpesvirus 6 DNA in acute lymphoblastic leukaemia. *Lancet* 1998; **352**:543–4.
8 Daibata M, Taguchi T, Nemoto Y *et al.* Inheritance of chromosomally integrated human herpesvirus 6 DNA. *Blood* 1999; **94**:1545–9.
9 Ward KN, Leong HN, Nacheva EP *et al.* Human herpesvirus 6 chromosomal integration in immunocompetent patients results in high levels of viral DNA in blood, sera, and hair follicles. *J Clin Microbiol* 2006; **44**:1571–4.
10 Tanaka N, Kimura H, Iida K *et al.* Quantitative analysis of cytomegalovirus load using a real-time PCR assay. *J Med Virol* 2000; **60**:455–62.

Key words: anticonvulsant hypersensitivity syndrome, chromosomal integration, drug eruption, drug-induced hypersensitivity syndrome, human herpesvirus 6

Conflicts of interest: none declared.

## Successful use of etanercept in type I pityriasis rubra pilaris

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Sir, Pityriasis rubra pilaris (PRP) is a rare, idiopathic, papulosquamous disease. It has many histological and clinical features in common with psoriasis and differentation of these two diseases is sometimes difficult. Systemic retinoids are the mainstay of treatment; however, PRP is often a therapeutic challenge. We describe a patient with type I PRP responding to etanercept. To the best of our knowledge, this is the first report of etanercept used successfully in PRP.

A 37-year-old man presented with a skin eruption of approximately 2 months' duration which was initially diagnosed as psoriasis. Narrowband ultraviolet (UV) B was commenced; however, after the first session, an increase in erythema developed which progressed into erythroderma. The patient underwent an urgent appendectomy after the second session of phototherapy. Dermatological examination revealed erythroderma with islands of sparing on the upper back, and marked palmoplantar keratoderma in association with thickened nails (Fig. 1a). Histopathological examination showed alternating orthokeratosis and parakeratosis in both vertical and horizontal directions as well as dilated hair follicle orifices and keratotic and parakeratotic plug formations which were consistent with PRP. Phototherapy was discontinued after three sessions. Routine biochemistry revealed mild elevations in liver enzymes [aspartate aminotransferase (AST) 56 IU $L^{-1}$ (normal 10–37), alanine aminotransferase (ALT) 85 IU $L^{-1}$ (normal 10–40)]. A 20-day trial of ciclosporin 3·5 mg $kg^{-1}$ daily was unhelpful. As liver enzymes had returned to normal during this time, acitretin was started at an initial dose of 0·3 mg $kg^{-1}$ daily which was later increased to 0·5 mg $kg^{-1}$ daily. Although 1 month of acitretin therapy led to only a slight improvement, it also resulted in progressive elevation of liver enzymes (AST 57 IU $L^{-1}$, ALT 125 IU $L^{-1}$) which persisted despite dose reduction. Hepatitis serology and autoimmune hepatitis markers were all negative, whereas hepatomegaly was detected in ultrasonographic examination. Owing to its hepatotoxic effects, acitretin was finally stopped 2 months after initiation. For the following 3 months, the patient was treated only with emollients and oral antihistamines. His erythrodermic condition remained unchanged during this period and was a great concern for the patient, but liver enzymes were normalized.

At this stage, etanercept was started at a dose of 50 mg twice a week. During the first 2 months of treatment, there was a slow regression in erythroderma and palmoplantar keratoderma which showed considerable improvement especially after the third month (Fig. 1b–d). This was followed by reducing the dose to 25 mg twice a week. The patient has been on etanercept for 6 months now and is almost clear. Treatment was well tolerated and no adverse effects were observed other than slight increases in liver enzymes without any need for treatment modification.

PRP usually has no preceding event. However, infection and UV radiation can be provocative.[1] Our patient underwent an urgent appendectomy concurrent with phototherapy. These two factors together may have contributed to the rapid progression of the disease.

PRP has been divided into five categories by Griffiths according to the age at onset, clinical presentation and natural

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