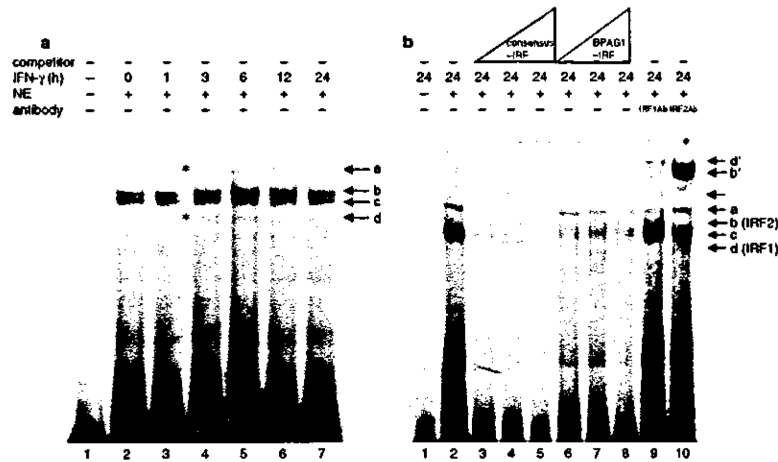

Figure 4. a) Electrophoretic mobility shift assay (EMSA) with nuclear extracts from unstimulated or stimulated normal human epidermal keratinocytes (NHEKs). Unstimulated nuclear extracts (lane 2) or extracts stimulated for 1 h (lane 3), 3 h (lane 4), 6 h (lane 5), 12 h (lane 6), and 24 h (lane 7) were incubated with the radiolabeled probe corresponding to consensus-interferon regulatory factor (IRF). Four complexes designated a, b, c, and d were detected after 3-h stimulation with interferon-γ (IFN-γ), and their concentration showed subsequent increases. b) The nuclear extracts stimulated with IFN-γ for 24 h were incubated with the radiolabeled probe corresponding to consensus–IRF. Competition experiments were carried out by adding unlabeled oligo, 50-fold, 100-fold, and 200-fold excess of consensus–IRF (lanes 3, 4, and 5, respectively) or 50-fold, 100-fold, and 200-fold excess of 230-kDa bullous pemphigoid antigen (BPAG1)–IRF (lanes 6, 7, and 8, respectively). Supershift experiments were performed by the addition of specific antibodies to either IRF1 (lane 9) or IRF2 (lane 10) to the binding reactions. Using consensus–IRF as a probe, four complexes designated a, b, c, and d after 3-h stimulation with IFN-γ were detected. In the competition experiments, all four complexes were competed out when non-radiolabeled consensus–IRF was added. When non-radiolabeled BPAG1–IRF was added, all four complexes were competed weakly. In the supershift experiments, the d complex was shifted in the presence of an antibody against IRF1, and the b complex was shifted in the presence of an antibody against IRF2. NE, nuclear extract.

induced in the extract of ≥3-h of IFN-γ treatment (Fig. 4a). To confirm the specificity of the binding activity, competition experiments with consensus–IRF probe were carried out by adding 50-fold, 100-fold, and 200-fold excess of non-labeled consensus–IRF oligo DNA, or 50-fold, 100-fold, and 200-fold excess of BPAG1–IRF. In the competition experiments, all these complexes were competed out when 200-fold excess of consensus–IRF oligo DNA was added (Fig. 4b). By adding BPAG1–IRF, which was identified in the BPAG1 promoter region, all these complexes were weakly competed out compared with consensus–IRF (Fig. 4b). The complex d was supershifted (band d′) in the presence of antibody against IRF1, and the complex b was supershifted (band b′) in the presence of antibody against IRF2, indicating that IRF1 and IRF2 were contained in band d and b, respectively.

Next, similar EMSA experiments were performed using BPAG1–IRF as a probe. NHEK were treated with 100 U/ml of IFN-γ for various periods, and nuclear extract was then isolated and incubated with the radiolabeled BPAG1–IRF probe. We found that one binding complex started to appear in the extract of 3-h IFN-γ treatment (indicated by arrow), and the intensity of this binding complex in the gel increased depending on time (Fig. 5a). In the competition experiments, the complex was significantly competed out when excess amount of non-labeled BPAG1–IRF was added. However, consensus–IRF showed weak, if any, competition to the binding (Fig. 5b). The supershift was not obtained in the presence of antibody against either IRF1 or IRF2 in this EMSA experiment of BPAG1–IRF (data not shown).

Discussion

Recent studies have revealed that IRFs expressed in the epidermal keratinocytes play crucial roles in the homeostasis of epidermal proliferation and differentiation, and aberrant IRF function is proposed to be involved in the pathogenesis of psoriasis vulgaris, a disorder of disorganized epidermal proliferation and differentiation, probably due to cytokines such as IFN-γ derived from infiltrating inflammatory cells (21).

Melany et al. (16) reported low expression of IRF1 in the psoriatic epidermis relative to its level in normal and uninvolved areas of the skin in the same patient. Furthermore, in the same study, cultured epidermal keratinocytes obtained from the skin of their patients showed poor response to IFN-γ for activation and induction of IRF1

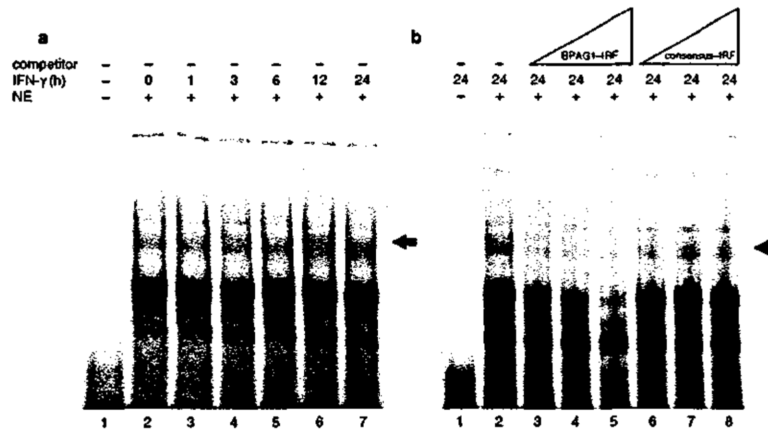

Figure 5. a) Electrophoretic mobility shift assay (EMSA) with nuclear extracts of cells unstimulated or stimulated with interferon-γ (IFN-γ). Unstimulated nuclear extracts (lane 2) or stimulated for 1 h (lane 3), 3 h (lane 4), 6 h (lane 5), 12 h (lane 6), and 24 h (lane 7) were incubated with the radiolabeled probe corresponding to 230-kDa bullous pemphigoid antigen (BPAG1)-interferon regulatory factor (IRF). One complex (arrow) was detected after 3-h stimulation with IFN-γ, and its concentration showed subsequent increase. b) The nuclear extracts stimulated by IFN-γ for 24 h were incubated with a radiolabeled probe corresponding to IRF-BPAG1. Competition experiments were carried out by adding unlabeled oligo, 50-fold, 100-fold, and 200-fold excess of BPAG1–IRF (lanes 3, 4, and 5, respectively) or 50-fold, 100-fold, and 200-fold excess of consensus–IRF (lanes 6, 7, and 8, respectively). The complex was competed out when non-radiolabeled BPAG1–IRF was added, but not competed out when non-radiolabeled consensus–IRF was added. NE, nuclear extract.

and STAT-1α (16). These findings suggest that abnormal signal transduction by IFN-γ is responsible, at least in part, for aberrant differentiation and proliferation of the psoriatic epidermis (21).

We studied epidermal gene regulation to uncover the molecular mechanisms of epidermal proliferation and differentiation. For this purpose, we selected BPAG1 promoter as a tool to clarify the molecular events responsible for maintaining undifferentiated basal keratinocytes phenotype, as BPAG1 is strictly expressed in the basal layer of the epidermis (22). In the course of the studies, we identified that IFN-γ transcriptionally down-regulated the promoter activity partly through the *cis*-element of ISRE–GAS chimeric sequence.

In the present study, we further investigated the IFN-γ-dependent regulation of BPAG1 gene, particularly the role of IRFs on the events. In the absence of IFN-γ stimulation, IRF1 mRNA expression was not detected in NHEK cultured with low calcium. This finding is in agreement with that reported by Nakanishi et al. (13). In contrast, IRF2 mRNA was detected in NHEK without IFN-γ in our repeated experiments, and previous study for IRF2 protein expression in the epidermis also indicated dominant expression of IRF2 as compared to IRF1 in the basal layer of the normal epidermis (23). Collectively, IRF2 seems to be constitutively expressed in cultured NHEK. Steady-state expression of IRF2 mRNA suggests the importance of IRF2 in the maintenance of the undifferentiating and proliferating basal keratinocyte phenotype. With IFN-γ stimulation, the balance between IRF1 and IRF2 mRNAs in NHEK was largely changed to an adverse expression pattern, a higher IRF1 expression compared with that of IRF2, probably because of a stronger response of *IRF1* gene to IFN-γ stimulation (24). Combined with the fact that the lower epidermis expresses IRF2 while the differentiated upper epidermis expresses higher IRF1 levels (23), the balance between IRF1 and IRF2 seems to play a key role in determining the impact of physiological and pathological states on epidermal proliferation and differentiation status.

The IRF–consensus sequence was identified by computer search in the promoter region from −135 to −123 of *BPAG1* gene, the expression of which is strictly restricted to the basal keratinocyte. Transient transfection studies with IRF1 and IRF2 expression vectors indicated that both IRF1 and IRF2 down-regulated BPAG1 promoter activity through the BPAG1–IRF site. These results indicate that IFN-γ stimulation down-regulates BPAG1 promoter activity through the induction of IRF1 and IRF2 in cultured NHEK and the existence of another transcription system that maintains BPAG1 gene expression in basal keratinocytes, which are known to express IRF2 constitutively.

Expression of IRF1 and IRF2 proteins in the cultured NHEK after IFN-γ stimulation was confirmed by gel morbidity shift assay with consensus IRF motif as a probe, and both IRF1 and IRF2 bindings on the probe were identified as supershifts with the addition of anti-IRF1 and anti-IRF2 monoclonal antibodies. The binding of IRF1 and

IRF2 to the consensus IRF was inhibited by the addition of BPAG1–IRF in the reaction, indicating BPAG1–IRF as a functional IRF motif. However, BPAG1–IRF did not show IRF1 and IRF2 binding in IFN-γ-treated NHEK nuclear extract but showed specific binding of another nuclear protein induced by IFN-γ. This binding was interfered with by the addition of consensus IRF, suggesting the existence of other IRF protein(s) with a higher affinity to BPAG1–IRF compared with IRF1 and IRF2, which is probably responsible for IFN-γ-induced down-regulation of the *BPAG1* gene.

Taken together, not only IRF1 and IRF2 but also other IRF family proteins could be induced by IFN-γ stimulation in the NHEK, and total balance of these IRF family proteins would modulate proliferation and differentiation of the keratinocytes in physiological and inflammatory skin conditions.

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All-*Trans*-Retinoic Acid Induces Interleukin-8 via the Nuclear Factor-κB and p38 Mitogen-Activated Protein Kinase Pathways in Normal Human Keratinocytes

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Retinoic acid derivatives have been used successfully for the treatment of various dermatoses, such as psoriasis; however, topical application of these compounds often elicits skin irritation. We hypothesized that this irritation was as a result of the local production of interleukin-8 (IL-8). To test this hypothesis, we investigated whether all-*trans*-retinoic acid (ATRA) induced IL-8 production in normal human keratinocytes. Stimulation with 10^{-7} M ATRA enhanced IL-8 mRNA expression and induced IL-8 production. We also studied the intracellular signaling mechanisms of ATRA-induced IL-8 production in keratinocytes. ATRA increased the expression of RelA (p65), RelB, nuclear factor (NF)-κB2 (p52), and NF-κB1 (p50), and elevated the DNA-binding activity of p65 and phosphorylation of inhibitor κB (IκB) α. Introduction of a dominant-negative mutant of IκBα completely abolished ATRA-induced IL-8 production, which indicates that this process is NF-κB-dependent. We also studied the role of the p38 mitogen-activated protein kinase (MAPK) pathway in this phenomenon. ATRA phosphorylated the p38 MAPK, and SB202180 inhibited ATRA-induced IL-8 production, which indicates that the p38 MAPK is also involved in ATRA-induced IL-8 production. In summary, ATRA induces IL-8 production in both NF-κB- and p38 MAPK-dependent manners in normal human keratinocytes.

Key words: skin irritation/chemokine/p65/retinoid/IκBα/SB202180
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Retinoids include natural and synthetic compounds with specific biological activity similar to that of Vitamin A (retinol) (Orfanos *et al*, 1997). Retinol and its derivative acid are widely used to treat acne, psoriasis, and photo-aged skin. Despite their many beneficial effects, however, topical application of retinoids often causes local irritation that is manifested as redness, scaling, and dryness (Kang *et al*, 1995; Ale *et al*, 1997). The mechanism of retinoic acid-induced inflammation is poorly understood. The important role of interleukin-8 (IL-8) in the progress of irritation has been demonstrated (Schroder, 1995). A recent report showed that treatment with 2% retinol upregulated the level of IL-8 mRNA in the ear epidermis of BALB/c mice (Kim *et al*, 2003), and that anti-irritants significantly inhibited retinoid-induced IL-8 in cultured keratinocytes (Kim *et al*, 2003), indicating that IL-8 plays a central role in retinoid-induced skin irritation. Although human keratinocytes produce IL-8 in response to retinoids (Kim *et al*, 2003), the molecular mechanism involved is unclear.

IL-8 is a member of the C–X–C subfamily of chemokines (CXCL8; Zlotnik and Yoshie, 2000). As a key factor in the pathogenesis of inflammatory diseases, IL-8 has diverse biological properties, including chemotaxis of neutrophils and T lymphocytes (Baggiolini *et al*, 1989), regulation of cell adhesion (Djeu *et al*, 1990), activation of neutrophils (Mukaida *et al*, 1992), and modulation of histamine release (Kuna *et al*, 1991). Therefore, it seems worthwhile to investigate the potential role of IL-8 in retinoic acid-induced skin inflammation. Various cell types, such as endothelial cells, epithelial cells, synovial cells, T cells, fibroblasts, keratinocytes, chondrocytes, and some tumor cells, have been shown to produce IL-8 (Hoffmann *et al*, 2002). The classical inducers of IL-8 are inflammatory stimuli, such as IL-1, tumor necrosis factor (TNF)-α, bacterial lipopolysaccharides (LPS), 12-*O*-tetradecanoylphorbol-13-acetate, viruses, and double-stranded RNA (Hoffmann *et al*, 2002). IL-8 production is regulated at both the transcriptional and post-transcriptional levels. The 3′-end of the IL-8 transcript contains an AU-rich *cis*-element (ARE), which is responsible for the destabilization of a variety of cytokine mRNA (Shaw and Kamen, 1986). The sequence that spans nucleotides (nt) −1 to −133 within the 5′-flanking region of the IL-8 gene is essential and sufficient for transcriptional regulation of the gene. The core IL-8 promoter contains a nuclear factor-κB (NF-κB) element that is required for activation in all of the cell types studied, and it also contains activating protein (AP)-1 - and CCAAT/enhancer-binding protein (C/EBP)-binding sites. The latter two sites are dispensable for transcriptional activation in some cell types, but contribute to

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Abbreviations: ATRA, all-*trans*-retinoic acid; CT, cycle threshold; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IκB, inhibitor κB; IL, interleukin; MAPK, mitogen-activated protein kinase; MOI, multiplicity of infection; NF-κB, nuclear factor κB; RAR, retinoic acid receptor; RARE, retinoic acid-responsive element; RXR, retinoid X receptor; TNF, tumor necrosis factor

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activation in other cell types. Thus, unlike the NF-κB site, the AP-1 and C/EBP sites are not essential for IL-8 gene induction, but are required for maximal gene expression (Hoffmann *et al*, 2002).

Cytokines and chemokines play important roles in the pathogenesis of various dermatoses. Based of the results of biochemical analyses and *in vitro* studies, it is clear that IL-8 contributes significantly to the pathological changes seen in psoriasis (Sticherling *et al*, 1991a, b, 1999; Takematsu and Tagami, 1993). The involvement of IL-8 in other dermatoses, such as bullous pemphigoid (Baroni *et al*, 2002), erythema migrans (Grygorczuk *et al*, 2002), atopic dermatitis, and allergic contact dermatitis (Albanesi *et al*, 2001), has also been demonstrated. The erythematous reaction in retinoid-induced skin irritation is clinically similar to a mild irritant dermatitis (Fisher *et al*, 1991; Ale *et al*, 1997; Grygorczuk *et al*, 2002). In particular, activated keratinocytes are an important source of chemotactic factors that direct the recruitment of specific leukocyte populations and regulate the quality, magnitude, and duration of the inflammatory response. Normal cultured human keratinocytes have been shown to produce IL-8 after appropriate stimulation (Larsen *et al*, 1989; Boorsma *et al*, 1994; Stoof *et al*, 2001).

Retinoic acid modulates immunological and inflammatory responses, most probably by regulating cytokine production, although it also affects epidermal cell growth and differentiation (Zitnik *et al*, 1994; Sawatsri *et al*, 2000). As retinoic acid upregulates IL-8 expression in fibroblasts (Zhang *et al*, 1992), neuroblastoma cells (Yang *et al*, 1993), and a human ovarian carcinoma cell line (Harant *et al*, 1995), we hypothesized that retinoic acid might also induce IL-8 production in human keratinocytes. To test this hypothesis, we investigated whether all-*trans*-retinoic acid (ATRA) was able to induce IL-8 production in cultured normal human keratinocytes, and we assessed the involvement of the NF-κB and p38 mitogen-activated protein kinase (MAPK) pathways in this process.

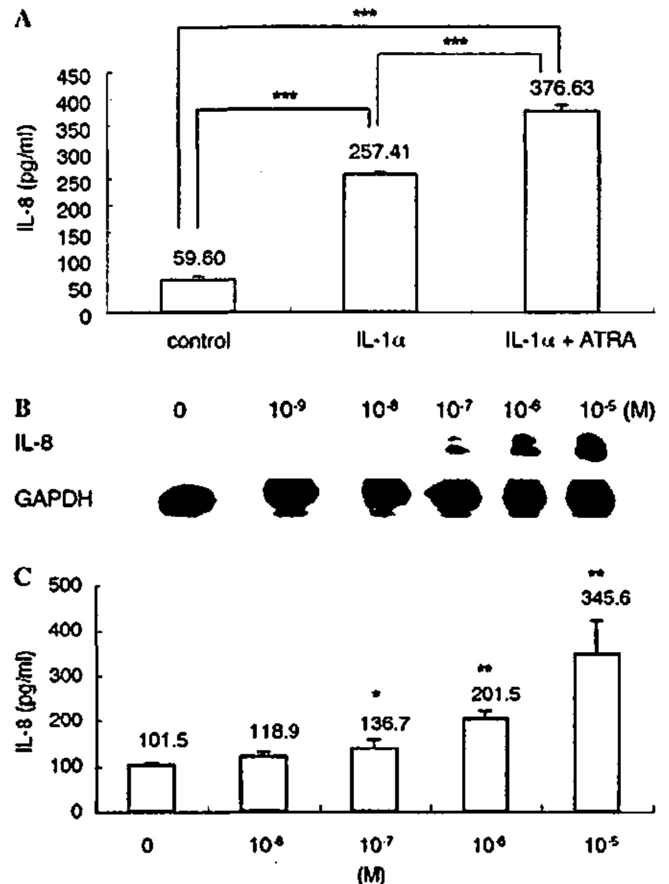


Figure 1
All-*trans*-retinoic acid (ATRA) simulates the production of interleukin (IL)-8 by human keratinocytes. (*A*) Subconfluent keratinocyte cultures were treated with IL-1α (1 ng per mL), ATRA (10^{-6} M), or vehicle (dimethyl sulfoxide). After 36 h of incubation, the culture supernatants were collected and subjected to ELISA for the detection of IL-8. (*B*) Keratinocytes were stimulated with ATRA (10^{-9}–10^{-5} M). IL-8 mRNA expression was monitored by RT-PCR after a 24-h incubation. (*C*) Keratinocytes were stimulated with ATRA for 36 h, and the level of secreted IL-8 was measured by ELISA.

Results

ATRA increases the production of IL-8 in keratinocytes IL-1α is the prominent inducer of IL-8 (Kristensen *et al*, 1991; Hoffmann *et al*, 2002). Moreover, retinoids reduce IL-1 induction of IL-8 production in human monocytes (Gross *et al*, 1993). Therefore, we initially investigated the effect of ATRA on IL-1α-induced IL-8 production. Keratinocytes were treated with IL-1α (1 ng per mL) or IL-1α plus ATRA (10^{-6} M), and the IL-8 levels in the culture supernatants were measured after 36 h of incubation. Marked induction of IL-8 was observed in IL-1α-stimulated cells, and ATRA increased IL-8 production in a synergistic manner (Fig 1*A*).

To assess the effect of ATRA on IL-8 production in keratinocytes, we stimulated the keratinocytes with different concentrations of ATRA. IL-8 mRNA expression was scarcely detectable by the ribonuclease protection assay (RPA) in samples that contained the vehicle (dimethyl sulfoxide (DMSO)) alone (Fig 1*B*). After 24 h of treatment with various doses of ATRA (10^{-9}–10^{-5} M), IL-8 mRNA expression was increased in a concentration-dependent manner (Fig 1*B*). The release of IL-8 into the culture medium was upregulated in a concentration-dependent manner, and was consistent with the increase in IL-8 mRNA (Fig 1*C*).

ATRA increases the expression of the NF-κB family members in human keratinocytes NF-κB binding is essential for the activation of IL-8 gene transcription (Kunsch *et al*, 1994). Several members of the NF-κB family, such as p50 (NF-κB1), p65 (RelA), c-Rel, and p52 (NF-κB2), have been shown to bind to the NF-κB motif of the IL-8 promoter (Kunsch and Rosen, 1993; Stein *et al*, 1993; Harant *et al*, 1996a). To characterize the molecular mechanisms underlying ATRA-induced IL-8 production in keratinocytes, the expression of NF-κB family members in ATRA-treated keratinocytes was examined using the RPA. The expression levels of p65, RelB, p52, and p50 mRNA started to increase 1 h after the addition of ATRA, and persisted for more than 24 h (Fig 2). Although c-Rel was detected faintly 12–48 h after ATRA treatment, the basal level of expression of c-Rel mRNA was very low in the RPA.

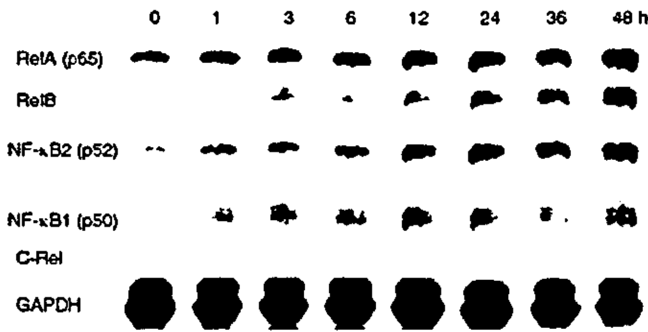
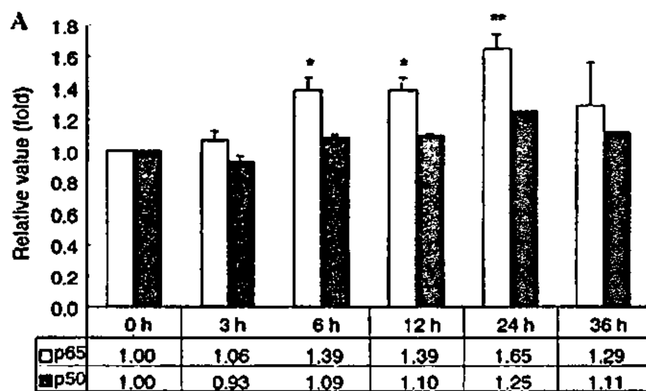


Figure 2
All-*trans*-retinoic acid (ATRA) increases the expression of nuclear factor (NF)-κB subunit mRNA. Keratinocytes were exposed to 10^{-6} M ATRA, and total RNA samples were collected at the indicated time points. The expression levels of the NF-κB subunit mRNAs were examined using the ribonuclease protection assay.

ATRA increases the DNA-binding activity of p65 in human keratinocytes We also examined the DNA-binding activities of the NF-κB family members in the nuclear extracts of ATRA-stimulated keratinocytes. The DNA-binding activity of p65 increased between 6 and 24 h post-treatment with ATRA (Fig 3*A*). In addition, ATRA upregulated the DNA-binding activity of p65 in a concentration-dependent manner (Fig 3*B*). In contrast, the DNA-binding activity of p50 was not influenced by ATRA. The DNA-binding activity of the c-Rel protein was not detected in either ATRA-treated or untreated keratinocytes (data not shown).

ATRA phosphorylates IκBα in cultured human keratinocytes The inhibitory molecule IκB anchors NF-κB in the inactive form in the cytosol (Baeuerle and Henkel, 1994). A crucial step in NF-κB signal transduction involves the dissociation of NF-κB from IκB, which is followed by NF-κB translocation to the nucleus (Baeuerle and Henkel, 1994).



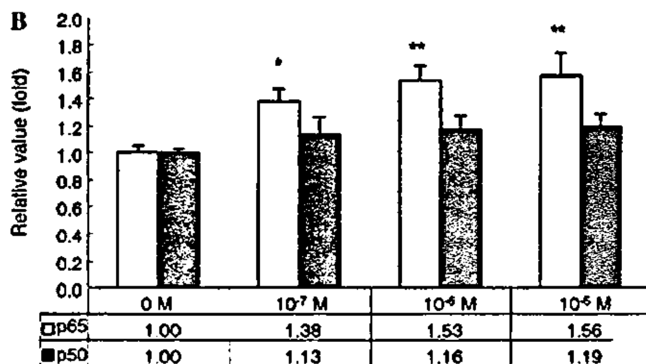


Figure 3
Nuclear translocation of p65 is increased by all-*trans*-retinoic acid (ATRA) treatment. (*A*) Keratinocytes were stimulated with ATRA (10^{-6} M), and the nuclear proteins were extracted at the indicated time points. An enzyme-linked immunoassay was performed to monitor the DNA-binding activities of the nuclear factor (NF)-κB subunits in the nucleus. The relative values for the optical density at 655 nm are normalized to the value of 0 h as 1 U, and are plotted on the graph. (*B*) ATRA (10^{-9}–10^{-5} M) was added to subconfluent-conditioned keratinocytes, and the cultures were incubated for 24 h. The DNA-binding activities of the NF-κB subunits in the nucleus were examined, and the relative values are normalized to the value for 0 M, which is designated as 1 U. *$p<0.05$; **$p<0.01$.

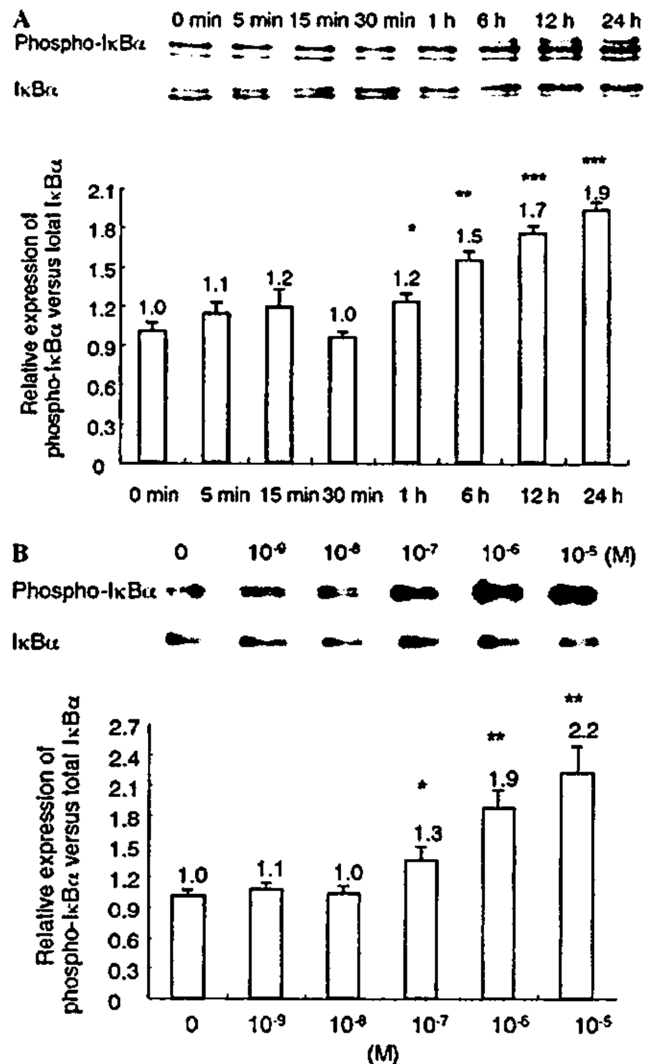


Figure 4
All-*trans*-retinoic acid (ATRA) increases the level of phosphorylated IκBα in human keratinocytes. (*A*) Keratinocytes were stimulated with ATRA (10^{-6} M), and the cellular proteins were extracted at the indicated time points. Immunoblotting was performed with anti-IκBα and anti-phospho-specific IκBα antibodies. The relative levels of phosphorylated IκBα are estimated using the total level of IκBα protein as the reference, and these values are normalized against the value at 0 min, which is designated as 1 U. (*B*) ATRA (10^{-9}–10^{-5} M) was added and incubated for 24 h. The relative levels of phosphorylated IκBα protein expression are estimated from the immunoblots. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

The dissociation of NF-κB from IκB requires the phosphorylation of IκB, which results in rapid and ubiquitous degradation of IκB. Thus, the effect of ATRA on the expression and phosphorylation of IκBα was investigated. ATRA increased the level of phosphorylation of IκBα, although ATRA had no significant effect on the total level of IκBα protein (Fig 4*A* and *B*). The relative level of expression of phosphorylated IκBα to total IκBα was increased significantly, 6 h post-ATRA treatment, and had increased 1.9-fold at 24 h (Fig 4*A*). In our concentration-dependency study, 24 h of ATRA treatment increased the level of phosphorylated IκBα 1.3-fold at 10^{-7} M, 1.9-fold at 10^{-6} and 2.2-fold at 10^{-5} M, as compared with vehicle-treated keratinocytes (Fig 4*B*).

Mutant IκBα abrogates the production of IL-8 by ATRA in human keratinocytes To determine whether ATRA-induced IL-8 production in keratinocytes was dependent on NF-κB, we introduced mutated-IκBα (IκBαM) gene using an adenovirus vector (AxIκBαM), which results in the blockade of NF-κB signals by unphosphorylated IκBα (Brown *et al*, 1995; Chen *et al*, 1995). Keratinocytes were transfected with AxIκBαM or AxLacZ, and stimulated with ATRA. The culture supernatants were collected at 12, 24, and 48 h after the addition of ATRA. The ELISA demonstrated that IκBαM not only abrogated ATRA-dependent production of IL-8, but also drastically suppressed the basal production of IL-8 (Fig 5*A*). IκBαM also suppressed IL-8 mRNA expression in both ATRA-treated and untreated keratinocytes (Fig 5*B*). The increased expression of IκBα protein in IκBαM-transfected keratinocytes confirms that mutated IκBα persists in the cell and suppresses the NF-κB signal (Fig 5*C*).

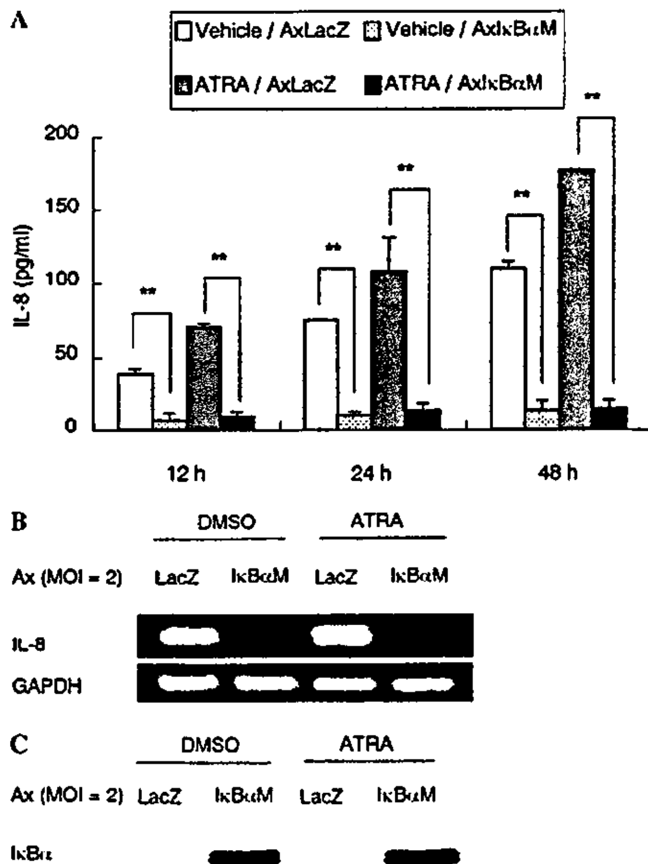


Figure 5
Blockade of nuclear factor (NF)-κB signaling abrogates the induction of interleukin (IL)-8 by all-*trans*-retinoic acid (ATRA). (*A*) Keratinocytes were transfected with AxIκBαM or AxLacZ at a multiplicity of infection (MOI) = 2 for 12 h before the addition of ATRA. ATRA (10^{-6} M) or vehicle was then added, and the cultures were incubated for the indicated time periods. The culture supernatants were collected, and the levels of IL-8 protein were measured. $^{*}p<0.05$; $^{**}p<0.01$. (*B*) Keratinocytes were transfected with adenovirus vector and stimulated with ATRA (10^{-6} M) or vehicle. The levels of IL-8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression 24 h post-treatment with ATRA were analyzed by RT-PCR. (*C*) Keratinocytes were transfected with adenovirus vector and stimulated with ATRA (10^{-6} M) or vehicle. The levels of IκBα protein expression 24 h after ATRA treatment were analyzed by western blotting.

The p38 MAPK inhibitor SB202180 partially suppresses ATRA-induced IL-8 expression in human keratinocytes We investigated whether the p38 pathway was also involved in ATRA-induced IL-8 gene expression in keratinocytes. After the addition of ATRA, the phosphorylation of p38 increased, whereas the total level of p38 protein was unchanged (Fig 6*A*). The phosphorylation of p38 started at 5 min, peaked at 1 h, and persisted for 24 h post-treatment with ATRA (Fig 6*A*). ATRA-dependent p38 phosphorylation was inhibited by SB202180 (Fig 6*B*). The cells were treated with 5, 10, or 20 μM of SB202180 for 2 h before the addition of ATRA, and the keratinocytes were then incubated for 36 h. ATRA-induced IL-8 production was inhibited in a concentration-dependent manner by SB202180 (Fig 6*C*). Consistent with the decrease in IL-8 production, the levels of ATRA-induced IL-8 mRNA expression were also decreased in a concentration-dependent manner by SB202180 (Fig 6*D*). In this study, SB202180 did not affect the basal level of IL-8 production (data not shown).

Discussion

In this study, we demonstrated that ATRA induces IL-8 production in cultured normal human keratinocytes. Skin irritation following topical application of retinoic acid at least partially explained by the local induction of IL-8 production by epidermal keratinocytes (Kim *et al*, 2003). IL-1α increases IL-8 production in normal human keratinocytes (Kristensen *et al*, 1991; Hoffmann *et al*, 2002), and ATRA increased IL-8 production with IL-1α synergistically (Fig 1*A*). This result is contrary to that of a previous report, in which IL-1-induced IL-8 production by human monocytes was markedly reduced by retinoids (Gross *et al*, 1993). Nevertheless, LPS-induced IL-8 production was increased by ATRA in a same cell system (Gross *et al*, 1993). In addition, the synergistic effects of ATRA and TNF-α and 12-*O*-tetradecanoylphorbol-13-acetate on IL-8 gene activation have been reported for the human melanoma cell line G-361 (Harant *et al*, 1996b) and the human acute promyelocytic leukemia cell line (Atkins and Troen, 1995), respectively. Therefore, the effects of ATRA on IL-8 production vary, according to both cell type and the co-administered reagent.

Retinoic acid exerts its pleiotropic effects through binding to two groups of nuclear receptors, the retinoic acid

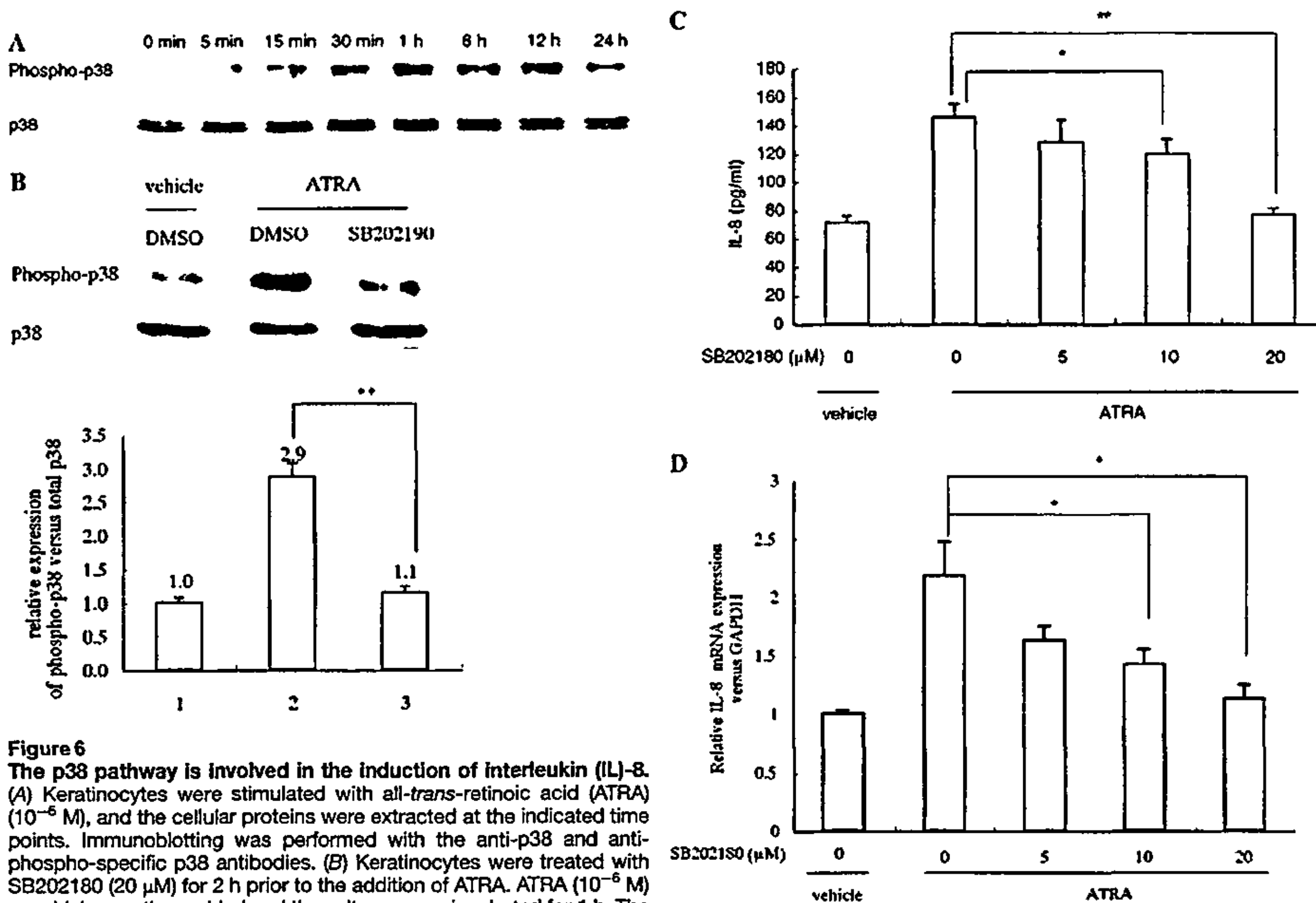


Figure 6
The p38 pathway is involved in the induction of interleukin (IL)-8. (*A*) Keratinocytes were stimulated with all-*trans*-retinoic acid (ATRA) (10^{-6} M), and the cellular proteins were extracted at the indicated time points. Immunoblotting was performed with the anti-p38 and anti-phospho-specific p38 antibodies. (*B*) Keratinocytes were treated with SB202180 (20 μM) for 2 h prior to the addition of ATRA. ATRA (10^{-6} M) or vehicle was then added and the cultures were incubated for 1 h. The expression levels of p38 and phospho-p38 were examined by western blotting. The intensity of each band was quantified as described in Materials and Methods, and the graph was made to show the significant effect of p38 inhibitor on ATRA-induced phospho-p38. (*C*) Keratinocytes were treated with various concentrations of SB202180 (0–20 μM) for 2 h. ATRA (10^{-6} M) or vehicle was then added and the cultures were incubated for 36 h. The levels of IL-8 in the culture supernatants were determined by ELISA. *$p<0.05$; **$p<0.01$. (*D*) Keratinocytes were treated with various concentrations of SB202180 (0–20 μM) for 2 h. ATRA (10^{-6} M) or vehicle was then added and the cultures were incubated for 18 h. The relative IL-8 mRNA expression levels were assessed by real-time quantitative RT-PCR.

receptors (RAR-α, -β, -γ) and the retinoid X receptors (RXR-α, -β, -γ) (Giguere *et al*, 1987; Petkovich *et al*, 1987; Benbrook *et al*, 1988; Brand *et al*, 1988; Krust *et al*, 1989). RAR usually form heterodimers with RXR (Chambon, 1996), and these dimers are able to bind to specific DNA sequences, which are known as retinoic acid-responsive elements (RARE). RAR–RXR complexes act as ligand-inducible transcription factors. The consensus sequence of RARE contains hexanucleotide half-sites that are arranged as inverted or direct repeats, spaced by various numbers of nt (de The *et al*, 1990); however, the IL-8 gene does not contain the classical ATRA response elements (Baggiolini and Clark-Lewis, 1992). Therefore, IL-8 induction by ATRA may not depend on this "classical pathway". In certain cases, retinoic acid has been shown to regulate genes that do not contain classical RARE motifs (Gudas *et al*, 1994). These genes can be regulated by retinoic acid via secondary events, such as the induction and activation of NF-κB (Segars *et al*, 1993). It has been shown that the induction of major histocompatibility class I genes by retinoic acid in human embryonal carcinoma NTera2 (NT-2) cells involves both the activation of RAR–RXR heterodimers and the induction of the NF-κB molecules p50 and p65 (Segars *et al*, 1993). The binding of NF-κB to the IL-8 promoter is essential for constitutive activation of IL-8 gene transcription (Hoffmann *et al*, 2002), and p65 is one of the major components responsible for κB binding to the IL-8 promoter (Kunsch and Rosen, 1993). ATRA is able to induce IL-8 gene expression by increasing NF-κB transcriptional activity in some cells (Harant *et al*, 1996a; Chang *et al*, 2000). In airway epithelium cells, ATRA-enhanced NF-κB binding to the promoter region involves p65 and p50 (Chang *et al*, 2000). In the human melanoma cell line G-361, the synergistic effect of ATRA and TNF-α on IL-8 expression is dependent on their combined activation of the IL-8 promoter, which requires an intact NF-κB-binding site (Harant *et al*, 1996b). In this study, we show nuclear translocation of p65 and IκB phosphorylation in ATRA-treated keratinocytes. Therefore, ATRA-dependent IL-8 induction probably involves increased levels of p65 (Segars *et al*, 1993). Similar effects of ATRA on NF-κB subunits have been observed in other cell types (Segars *et al*, 1993; Feng and Porter, 1999; Farina *et al*, 2002). ATRA-mediated increases in the levels of p65 and p50 transcripts are dependent on the "classical pathway".

An alternative mechanism for IL-8 induction via NF-κB signaling is the phosphorylation of IκBα. Unfortunately, the molecular mechanism by which ATRA phosphorylates IκBα is unclear. The introduction of IκBαM completely abolished

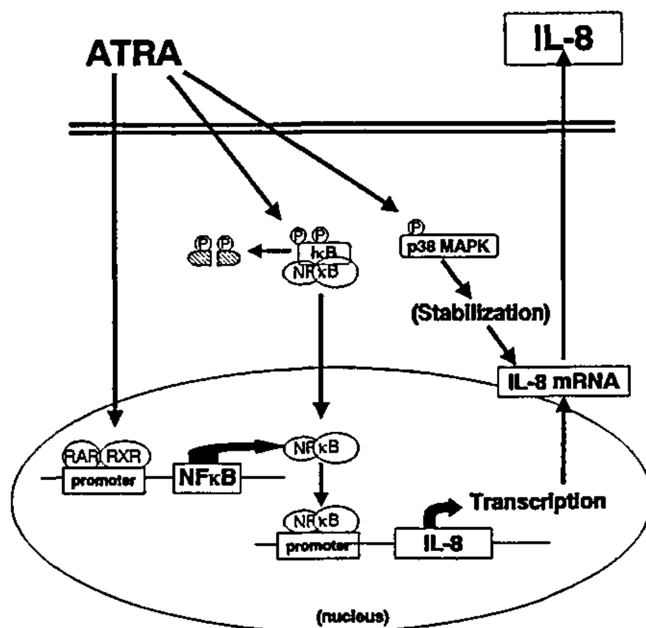


Figure 7
Schematic representation of a possible mechanism for all-*trans*-retinoic acid (ATRA)-dependent induction of interleukin (IL)-8 production by keratinocytes. A possible mechanism for ATRA-dependent induction of IL-8 production by keratinocytes is presented. ATRA phosphorylates IκBα, which causes nuclear translocation of nuclear factor (NF)-κB elements, and results in the transactivation of the NF-κB signal. The increased levels of p65 and p50 induced by ATRA may also contribute to activation of the NF-κB signal. Activation of the p38 mitogen-activated protein kinase (MAPK) pathway may play a role in IL-8 mRNA stabilization and IL-8 production.

IL-8 production and reduced the basal level of IL-8 production. These findings suggest that NF-κB activation is essential in ATRA-induced IL-8 production in normal keratinocytes.

Activation of p38 MAPK was elicited by ATRA in keratinocytes. The finding that the p38 inhibitor SB202180 specifically decreases ATRA-induced IL-8 production suggests that the p38 MAPK is also involved in ATRA-induced IL-8 production. A previous report has demonstrated that the p38 MAPK pathway contributes to cytokine/stress-induced IL-8 gene expression by stabilizing mRNA through an ARE-targeted mechanism at the post-transcriptional level (Hoffmann *et al*, 2002). Therefore, ATRA may stabilize IL-8 mRNA by activating the p38 pathway, thereby enhancing IL-8 production in keratinocytes. In summary, ATRA induces IL-8 production in normal human keratinocytes via NF-κB and the p38 MAPK signal pathways.

Materials and Methods

Cell culture Normal human keratinocytes were cultured in MCDB153 medium that was supplemented with insulin (5 μg per mL), hydrocortisone (5×10^{-7} M), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine hypothalamic extract (50 μg per mL), and Ca^{2+} (0.03 mM), as described previously (Yamasaki *et al*, 2003a). The study was conducted according to Declaration of Helsinki principles. All of the procedures that involved human subjects received prior approval from the Ethical Committee of Ehime University School of Medicine, and all the subjects provided written informed consent.

Reagents ATRA was purchased from Sigma Chemical Co (St Louis, Missouri), and was dissolved in DMSO. Recombinant human IL-1α was a generous gift from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). The anti-IκBα and anti-phospho-IκBα antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, Massachusetts). The p38 MAPK inhibitor SB202180 was purchased from Calbiochem-Novabiochem International Co. (San Diego, California), dissolved in DMSO at a concentration of 2 mM, and stored at −20°C.

Adenovirus construction and infection The cosmid cassette pAxCAw (Miyake *et al*, 1996) and the parental virus Ad5-dlX (Miyake *et al*, 1996) were kind gifts from Dr Izumu Saito (Tokyo University, Japan). The full-length coding region of the IκBαM cDNA was obtained from the *Hin*dIII- and *Bam*HI-digested fragments of pCMV-IκBαM (BD Biosciences Clontech, Palo Alto, California), and subcloned into the cosmid cassette pAxCAw. IκBαM encodes the dominant negative mutant form of IκBα, which is a non-degradable form of human IκBα, in which serines 32 and 36 are replaced by alanine residues (S32A/S36A), thereby blocking its phosphorylation and degradation (Brown *et al*, 1995; Chen *et al*, 1995). The NF-κB signal cannot be activated in cells that over-express IκBαM, regardless of the presence of extracellular stimuli that normally induce the phosphorylation of IκBα and activation of the NF-κB signal (Brown *et al*, 1995; Feig *et al*, 1999). An adenovirus vector that contains the CAG promoter and IκBαM (AxIκBαM) was generated using the cosmid cassettes and Ad DNA-TPC (COS-TPC) method, and virus stocks were prepared using the standard procedure (Miyake *et al*, 1996). Concentrated, purified virus stocks were prepared by CsCl gradient centrifugation, and the virus titer was estimated in a plaque formation assay.

Subconfluent cultures of normal human keratinocytes were infected with AxIκBαM at a multiplicity of infection (MOI) of 2, and AxLacZ was used as the control vector. The vectors were cultured with the cells for 60 min, with a brief period of agitation every 15 min. This was followed by the addition of fresh culture medium, and re-incubation for 12 h, before treatment with ATRA. The expression of these genes was confirmed by RT-PCR using specific probes.

Oligonucleotide probe preparation PCR-amplified human cDNAs were inserted into the *Eco*RI and *Hin*dIII sites of the pPMG vector (BD Pharmingen, San Diego, California). The inserted cDNA corresponded to the following sequences: nt 412–746 of RelA (p65) (GenBank/EBI accession no. NM021975); nt 709–1013 of RelB (NM006509); nt 804–1074 of NF-κB2 (p52/p100) (NM002502); nt 877–1122 of NF-κB1 (p50/p105) (NM003998); nt 1758–1982 of c-Rel (X75042). The pPMG vector (Pharmingen), which incorporates the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, was used as the internal standard.

RPA Total RNA was isolated from cultured human keratinocytes using Isogen (Nippon Gene, Toyama, Japan). Single-stranded antisense riboprobes were prepared by *in vitro* transcription of human cDNA fragments using the RiboQuant *In Vitro* Transcription Kit (Pharmingen) in the presence of [α-^{32}P] UTP. The hCK-5 probe (Pharmingen) for IL-8 detection and an oligonucleotide probe for NF-κB were used as the templates for the *in vitro* transcription reaction. The hybridization products were separated on a gel, and exposed to photographic film, as described previously (Yamasaki *et al*, 2003b). At least three independent studies were performed, with similar results. One representative experiment is shown in each figure. The GAPDH bands appear as a doublet, as reported previously (Dai *et al*, 2004), which may be because of the fact that the end of the GAPDH mRNA is highly susceptible to RNase digestion, even though it is double stranded.

RT-PCR Total RNA samples from cultured cells were isolated at the indicated time points using Isogen (Nippon Gene Co.). RT-PCR was performed using RT-PCR High Plus (Toyobo, Osaka, Japan), according to the manufacturer's instructions. The cDNA was gen-

erated from reverse transcription of total RNA for 30 min at 60°C, and then heated to 94°C for 2 min. The PCR cycle consisted of 1 min at 94°C for denaturation, and 1.5 min at 60°C for annealing and primer extension. The IL-8 sequences were amplified for 34 cycles, and the GAPDH sequences for 22 cycles. The following primer pairs were used: human IL-8, 5'-CTTCTCTGCAGCACATCC-3' and 5'-AAGACCTCTCAAGGCTTTG-3'; and GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. The PCR products were visualized on 2% agarose gels that contained ethidium bromide. The PCR products were sequenced, to confirm the accuracy of amplification.

Real-time quantitative PCR Total RNA samples from cultured cells were isolated using Isogen (Nippon Gene Co.). Real-time RT-PCR was performed in the ABI PRISM 7700 sequence detector (PE Applied Biosystems, Branchburg, New Jersey). The Pre-Developed TaqMan assay reagents (set of primers and probe) for human IL-8 (Accession Numbers: NM_000584) and for human GAPDH endogenous control (Accession Numbers: NM_002046) were purchased from Applied Biosystems (Foster City, California). The RNA analysis was undertaken using the TaqMan One-Step RT-PCR Master Mix reagents kit (PE Applied Biosystems) and 500 ng of total RNA. Thermal cycling was initiated at 48°C for 30 min for reverse transcription reaction, followed by a first denaturation step at 95°C for 10 min, and then 40 cycles PCR at 95°C for 15 s and at 60°C for 1 min according to the manufacturer's instructions using 2 × AmpliTaq Gold DNA Polymerase mix (25 μL), 40 × RT enzyme mix (1.25 μL), 20 × Pre-Developed TaqMan assay reagent (2.5 μL). All one-step RT-PCR reactions were performed in 50 μL reaction volumes, and carried out in a 96-well plate. This experiment used two dye layers to detect the presence of target and control sequences. The 6-FAM dye layer yielded the results for quantification of IL-8 mRNA, and the VIC dye layer yielded the results for quantification of GAPDH. Quantification of gene expression was performed using the comparative cycle threshold (CT) method (Sequence Detector User Bulletin 2; Applied Biosystems) and reported as the fold difference relative to GAPDH gene. To calculate the fold change (increase or decrease), the CT of the housekeeping gene (GAPDH) was subtracted from the CT of the target gene (IL-8) to yield the ΔCT. Change in expression of the normalized target gene as a result of an experimental manipulation was expressed as $2^{-\Delta\Delta CT}$ where $\Delta\Delta CT = \Delta CT$ sample$-\Delta CT$ control as described previously (Babu and Nutman, 2003). In this study, each assay was performed in triplicate, and the fold change of each sample was normalized against that of the vehicle as 1 U.

ELISA Culture supernatants were collected at the indicated time points after treatment, and stored at −70°C until used for ELISA. The IL-8 ELISA kit was purchased from R&D Systems (Minneapolis, Minnesota), and used according to the manufacturer's instructions. The optical density at 450 nm was measured with an Immuno Mini NJ-2300 microplate reader (Nalge Nunc International K.K., Tokyo, Japan). All of the assays were performed at least three times.

Western blot analysis The cells were harvested by scraping into extraction buffer that contained 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 7.4), and protease inhibitors. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. The analysis was performed using the Vistra ECF Kit (Amersham Biosciences K.K., Tokyo, Japan) and the FluoroImager (Molecular Dynamics Inc., Sunnyvale, California), as described previously (Yamasaki *et al*, 2003b). At least three independent studies were performed, with similar results. One representative experiment is shown in each of the figures. The intensity of each band was quantified using ImageQuant (Molecular Dynamics Inc.), with reference to the control signal, which was assigned the value of 1 U. Reproducibility was confirmed by performing three independent experiments.

Nuclear protein preparation and detection of transcription factor activity Nuclear proteins were prepared according to the instructions provided in the TransFactor Extraction Kit (BD Biosciences Clontech, Palo Alto, California). Briefly, cells were harvested in lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT) that contained protease inhibitors. Nuclear pellets were collected by centrifugation, and resuspended in extraction buffer (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 1 mM DTT) that contained protease inhibitors. Finally, the protein concentrations of the nuclear extracts were determined, and the samples were stored at −70°C until used.

DNA-binding activities of the NF-κB family members were determined using the BD Mercury Transfactor Kits (BD Biosciences Clontech), according to the manufacturer's instructions. The relative optical density values at 655 nm were normalized to the control values (at 0 h or for 0 M), which were designated as 1 U, and plotted on a graph. All of the assays were performed on at least three separate occasions.

Statistical analyses The data were collected from at least three independent experiments. Quantitative data are expressed as the mean ± SE. Statistical significance was determined by the paired Student's *t* test. Differences were considered to be statistically significant for $p<0.05$. The levels of statistical significance are indicated, as follows, in the figures: $^{*}p<0.05$; $^{**}p<0.01$; and $^{***}p<0.001$.

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TGF-β is not involved in early phase growth inhibition of keratinocytes by 1α,25(OH)$_2$vitamin D$_3$

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Growth inhibition;
RNase protection assay

Summary

Background: It has been proposed that transforming growth factor-β (TGF-β) is involved in the growth inhibition of normal human epidermal keratinocytes (NHEK) by 1α,25-dihydoxyvitamin D$_3$ (1α,25(OH)$_2$D$_3$), although this is still controversial because of the difficulty in blocking TGF-β activity completely.
Objective: To determine whether TGF-β is involved in early phase growth inhibition by 1α,25(OH)$_2$D$_3$.
Methods: TGF-β mRNA was detected by ribonuclease protection assay (RPA), and biological active TGF-β was determined by a luciferase reporter assay. To block intrinsic TGF-β activity completely, we constructed an adenovirus vector expressing a truncated TGF-β type II receptor with a dominant negative effect (*AdexTβTR*) that blocks TGF-β signal transduction.
Results: 1α,25(OH)$_2$D$_3$ slightly upregulated TGF-β1 and TGF-β2 after 24 h according to an RPA and a luciferase reporter assay, however growth inhibition by 1α,25(OH)$_2$D$_3$ occurred at 6 h. The addition of 10^{-6} M of 1α,25(OH)$_2$D$_3$ to NHEK infected with *AdexTβTR* or *AdexLacZ* (control vector) reduced DNA synthesis to 59.3 and 62.2% at 6 h, respectively. There was no *significant* difference in cell number after a 3-day incubation with *AdexTβTR* or *AdexLacZ*-infected cells treated with 1α,25(OH)$_2$D$_3$.

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Conclusion: Since $1\alpha,25(OH)_2D_3$ rapidly inhibits NHEK growth regardless of the prevention of TGF-β signal transduction, TGF-β is not involved in early phase growth inhibition by $1\alpha,25(OH)_2D_3$.

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1. Introduction

1α,25-dihydoxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) is the active form of vitamin D_3. It plays roles in many biological phenomena, such as calcium homeostasis and bone formation [1]. The binding of $1\alpha,25(OH)_2D_3$ to the nuclear receptors for the hormonally active form of vitamin D activates the vitamin D receptor (VDR), which subsequently regulates physiological events, such as cellular differentiation and proliferation [2–5]. $1\alpha,25(OH)_2D_3$ has been shown to decrease the proliferation of normal human epidermal keratinocytes (NHEK) [6–8]. NHEK possess receptors for vitamin D_3, including the VDR and retinoid X receptors [9,10]. $1\alpha,25(OH)_2D_3$ is reported to be effective in the treatment of psoriasis [11,12]. However, the precise mechanism of action of $1\alpha,25(OH)_2D_3$ in inhibiting NHEK proliferation is not clear.

The transforming growth factor-β (TGF-β) family consists of isoforms that include TGF-β, bone morphogenetic protein, and activin [13,14]. TGF-β is a 25 kDa peptide that has inhibitory effects on NHEK proliferation [15,16]. It stimulates fibroblast growth and the production of extracellular matrix materials like collagen, integrin, and plasminogen activator inhibitor-1. Three different isoforms of TGF-β have been described in mammalian cells, and the expression of these isoforms is regulated in different manners [17]. TGF-β members transduce signals by simultaneously contacting two transmembrane serine/threonine kinases called type I and type II receptors [18]. The type II receptor for TGF-β binds the ligand, whereas the type I receptor does not. Type I receptors recognize ligand-bound type II receptors, and these are thought to form a heterotetramer. A central event in TGF-β signal transduction is phosphorylation of the type I receptor, which is catalyzed by the type II receptor kinase domain. A mutant type II receptor, in which the kinase domain is truncated, is known to block the growth inhibitory effect of TGF-β [19].

TGF-β, which is an autocrine growth inhibitor for NHEK, is thought to be involved in the growth inhibition by $1\alpha,25(OH)_2D_3$ or $1,25(OH)_2D_3$ [20,21], although this is still controversial because of the difficulty in blocking endogenous TGF-β activity in NHEK completely. To solve this problem, we constructed a replication-deficient adenovirus vector expressing a truncated serine/threonine kinase domain of the TGF-β type II receptor with a dominant negative effect that blocks TGF-β signal transduction completely. Using this adenovirus vector system to introduce foreign genes into all keratinocytes and abolish endogenous TGF-β signaling completely, we analyzed whether TGF-β is involved in growth inhibition by $1\alpha,25(OH)_2D_3$.

2. Materials and methods

2.1. Materials

$1\alpha,25(OH)_2D_3$ was a generous gift from the Teijin Pharmaceutical Co., Ltd. (Tokyo, Japan). Recombinant human TGF-β1 and TGF-β2 were purchased from R&D, Inc. (Minneapolis, MN).

2.2. Cell culture

Normal human epidermal keratinocytes were cultured with MCDB153 medium supplemented with insulin (5 μg/ml), hydrocortisone (1 μM), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine hypothalamic extract (BHE) (50 μg/ml), and Ca^{2+} (0.1 mM) as described previously [22,23]. Third or fourth passage cells were used in this study.

2.3. Measuring bromodeoxyuridine (BrdU) uptake

NHEK were seeded on 24-well plates at a density of 8 $\times 10^4$ cells/well. After reaching subconfluency, the cells were fed with MCDB153 medium lacking BHE. The following day, the cells were fed once again with the same medium containing 1×10^{-6} M of $1\alpha,25(OH)_2D_3$ and incubated for 6, 12, or 24 h. The cells were then incubated with medium containing BrdU for 2 h. BrdU incorporation was determined using a cell-proliferating ELISA system (Amersham Life Science Inc., Arlington Heights, IL) according to the manufacturer's instructions. The absorbance at

450 nm was measured using a spectrophotometer (Pharmacia Inc., Uppsala, Sweden).

2.4. RNase protection assay

Analysis was performed using a multiprobe RNase protection assay system (PharMingen, BD PharMingen, San Diego, CA) according to the manufacturer's instructions. Ten micrograms of total RNA were hybridized with ^{32}P-labeled riboprobe and digested with RNase. The hybridization products were separated on a 5% polyacrilamide/8 M urea gel and exposed to film (BioMax MS, Kodak Co., Rochester, NY). GAPDH was used as an internal standard. The intensity of each band was quantified using Diversity Database™ software.

2.5. Construction of replication-deficient recombinant adenoviruses

A replication-deficient E1- and E3-recombinant adenovirus vector expressing the truncated human type II TGF-β receptor was prepared as described previously [19]. Briefly, human type II TGF-β receptor cDNA with most of its cytoplasmic kinase region deleted (only 7 amino acids remained in the intracellular region) was tagged with an influenza virus hemagglutinin (HA) epitope at its N-terminus. This was placed into a cassette cosmid vector, pAdexCA1w (provided by Izumu Saito, University of Tokyo) under a CA promoter consisting of a cytomegalovirus enhancer and a chicken β-actin promoter (*pAdexTβTR*). A recombinant adenovirus was constructed by in vitro homologous recombination in 293 cells using *pAdexTβTR* and the adenovirus DNA-terminal protein complex by a method previously described. The desired recombinant adenovirus, designated *AdexTβTR*, was purified by cesium chloride ultracentrifugation followed by extensive dialysis. The titer of the virus stock solution was assessed by a plaque formation assay using 293 cells and expressed in plaque-forming units. Two control adenovirus vectors were used: *AdexLacZ* expressing bacterial β-galactosidase and *Adex1w* containing no exogenous gene. Transfection of NHEK with adenovirus vectors was carried out by incubating subconfluent cells with the vectors in MCDB153 medium lacking BHE for 90 min at 37 °C with gentle rocking every 15 min. Then, the medium was exchanged with MCDB153 medium and the cells were incubated at 37 °C in 5% CO_2 until assays.

2.6. Western blot analysis

Subconfluent NHEK infected with *AdexTβTR* were lysed in radioimmunoprecipitation buffer (50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 mM EDTA, 10 mM Tris, pH 7.4, 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 0.2 unit/ml aprotinin, 10 mM pepstatin A, and 25 mM leupeptin). The lysates were purified with wheat germ agglutinin-Sepharose beads (Amersham Pharmacia Biotech Ltd., Buckinghamshire, UK), subjected to 12% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Amersham). Each membrane was blocked with 5% non-fat dry milk in Tris–HCl pH 7.4, 0.15 M NaCl, 0.05% Tween-20, and soaked with a 1:1000 dilution of anti-HA antibody (12CA5). After washing, the membrane was incubated with 1:2500 fluorescein-labeled goat anti-mouse IgG for 1 h. The signal was amplified with an anti-fluorescein alkaline phosphatase conjugate followed by the fluorescent substrate AttoPhos (Amersham). The membrane was scanned using FluoroImager (Molecular Dynamics Inc., Sunnyvale, CA).

2.7. Luciferase reporter assay

A reporter plasmid containing the TGF-β/Smad responsive element and firefly luciferase (*p3TP-lux*) was a generous gift from Dr. Kohei Miyazono (Tokyo University, Japan). To normalize the transfection efficiency, a plasmid containing Renilla luciferase driven by the cytomegalovirus promoter (pRL-CMV) was included in the assay. NHEK were seeded at a density of 4×10^4 cells per well in 12-well collagen-coated plates in 1 ml of MCDB153 medium with BHE. The medium was replaced 48 h later with 1 ml of fresh MCDB153 with BHE, and *AdexTβTR*, *Adex1w* or *AdexLacZ* were transfected (MOI = 5). After 24 h of adenovirus infection, 1 μg of *p3TP-lux* and 0.1 μg of *pRL-CMV* were transfected using *FuGENE6* (Japan Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. *p3TP-lux* contains three tandem repeats of a TGF-β-signal responsive element, which is activated by TGF-β stimulation [24,25]. After 24 h, the cells were treated by the addition of $1\alpha,25(OH)_2D_3$ (10^{-6} M), and incubated further. Then, the cells were harvested at the indicated times with 200 μl of lysis buffer (Promega Corporation, Madison, WI), and the luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega) with a luminometer (Luminescencer JNR AB-2100, Atto Co., Osaka, Japan). Transfection was performed in triplicate. The relative luciferase activity was calculated by normalizing it to the Renilla luciferase activity. Statistical analysis was performed using Student's *t*-test.

2.8. Cell cycle analysis

The cell cycle distribution was analyzed using a CycleTEST™ PLUS DNA reagent kit (Becton Dickinson Immunocytometry Systems, San Jose, CA) according to the manufacturer's instructions. Nuclei isolated by trypsinization were stained with propidium iodide and then run on a flow cytometer (FACSCalibur, Becton Dickinson Biosciences, San Jose, CA).

3. Results

3.1. $1\alpha,25(OH)_2D_3$ upregulates TGF-β1 and TGF-β2 mRNA in NHEK

To investigate whether $1\alpha,25(OH)_2D_3$ upregulates the expression of TGF-β isoform mRNA in normal human epidermal keratinocytes, 10^{-6} M $1\alpha,25(OH)_2D_3$ was added to the medium, and total RNA was harvested at the indicated times, and subjected to an RNase protection assay. $1\alpha,25(OH)_2D_3$ gradually increased TGF-β1 mRNA expression from 3 to 24 h. Maximal induction was observed at 24 h, and was 3 times control levels. $1\alpha,25(OH)_2D_3$ also upregulated TGF-β2 mRNA from 3 to 6 h after stimulation. Optimal induction occurred at 3 h, and was increased 4-fold; this was followed by a rapid reduction to the steady-state level (Fig. 1A, B). TGF-β3 mRNA was not altered by $1\alpha,25(OH)_2D_3$.

3.2. Adenovirus-mediated expression of the truncated human type II TGF-β receptor in keratinocytes

We examined the expression of the truncated human type II TGF-β receptor in NHEK infected by *AdexTβTR* at various multiplicity of infection (MOI). Subconfluent NHEK in 6 cm dishes were incubated with *AdexTβTR* at MOI = 5, 10 or 20 for 90 min in 3 ml of MCDB 153 medium lacking BHE. Medium was changed to MCDB153 medium and cultured further. After 24 h, cell lysates were harvested and subjected to Western blot analysis. Western blots showed a glycosylated protein of approximately 45 kDa corresponding to the truncated human type II TGF-β receptor using an antibody against the HA epitope (Fig. 2A). The expression level was MOI-dependent. Next, we examined the time course of its expression at an MOI of 5. The truncated human type II TGF-β receptor protein was detected at 6 h, and submaximal expression was achieved at 24 h after infection (Fig. 2B).

3.3. AdexTβTR suppressed the TGF-β1 or TGF-β2-induced growth inhibition of NHEK

Since a 90 min infection with *AdexTβTR*-induced the expression of mutant *TβTR* in NHEK after 24 h, we examined whether mutant *TβTR* could inhibit TGF-β action on NHEK. NHEK were seeded on 24-well plates at a density of 8 × 10^4 cells/well. After

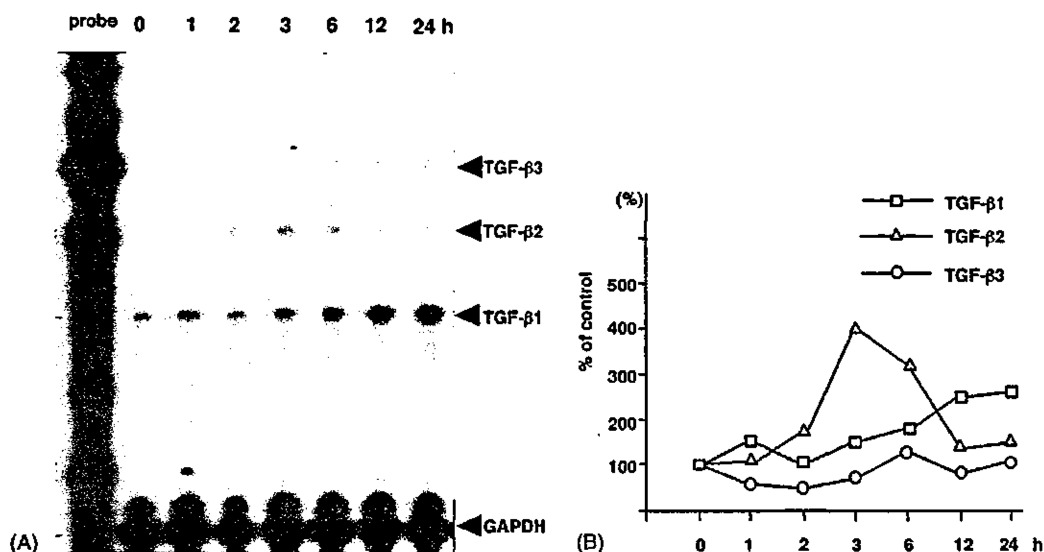

Fig. 1 Effect of $1\alpha,25(OH)_2D_3$ on the expression of TGF-β1, 2, and 3 in NHEK. (A) Subconfluent keratinocytes were incubated with 1 × 10^{-6} M $1\alpha,25(OH)_2D_3$ and total RNA was extracted at the indicated times using Isogen (Nippon Gene, Tokyo, Japan). Ten micrograms of total RNA were hybridized with ^{32}P-labeled riboprobe and digested with RNase. The hybridization products were separated on a 5% polyacrilamide/8 M urea gel and exposed to film (Kodak BioMax MS). GAPDH is shown as an internal standard, (B) The intensity of each band was quantified using Diversity Database™ software, and the relative values of TGF-β1, TGF-β2 and TGF-β3 mRNA were plotted on graphs.

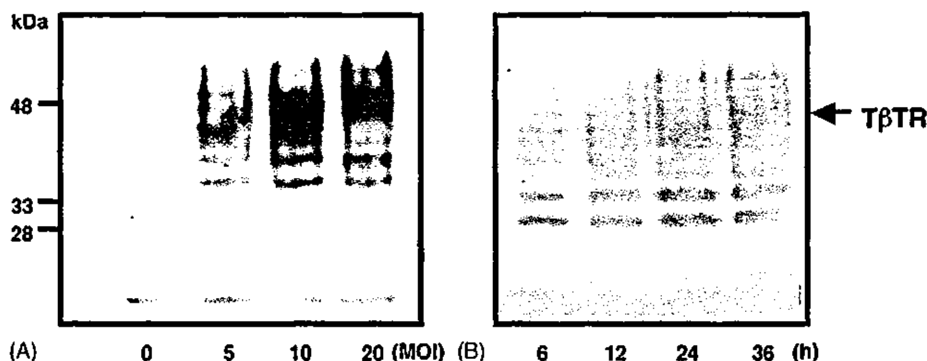

Fig. 2 Adenovirus-mediated expression of the truncated type II TGF-β receptor in normal human keratinocytes, (A) Cell lysates were harvested 24 h after infection with *AdexTβTR* at the indicated multiplicity of infection (MOI), subjected to SDS-PAGE electrophoresis, and transferred to a membrane. The membrane was probed with a monoclonal antibody against HA epitope, and the signal was visualized using fluorescein-conjugated anti-mouse IgG, and scanned using FluoroImager, (B) Cell lysates were harvested at the indicated times after infection with *AdexTβTR* at an MOI of 5. Truncated type II TGF-β receptor was detected at 6 h, and maximal expression was achieved 24 h after infection.

reaching subconfluency, the cells were fed with MCDB153 medium lacking BHE and *AdexTβTR* or *Adex1w* was infected at various MOI. After 24 h, medium was replaced with fresh MCDB153 and recombinant human TGF-β1 or TGF-β2 was added to the well at various concentrations. After 24 h, the cells were then incubated with medium containing BrdU for 2 h. BrdU incorporation was determined using a cell-proliferating ELISA system (Amersham Life Science Inc.) according to the manufacturer's instructions. TGF-β1 and TGF-β2 decreased DNA synthesis from 0.1 to 1 ng/ml in the *Adex1w*-infected cells, whereas in the *AdexTβTR*-treated cells, DNA synthesis was not suppressed by the addition of exogenous TGF-β1 or TGF-β2 (Fig. 3).

3.4. 1α,25(OH)$_2$D$_3$ increased biologically active TGF-β after 24 h

To identify the increase in biologically active TGF-β, we examined TGF-β/Smad-signal-responsive element activity using a *luciferase reporter assay.* NHEK were infected by *AdexTβTR* or *AdexLacZ* and then co-transfected with *p3TP-lux* and *pRL-*

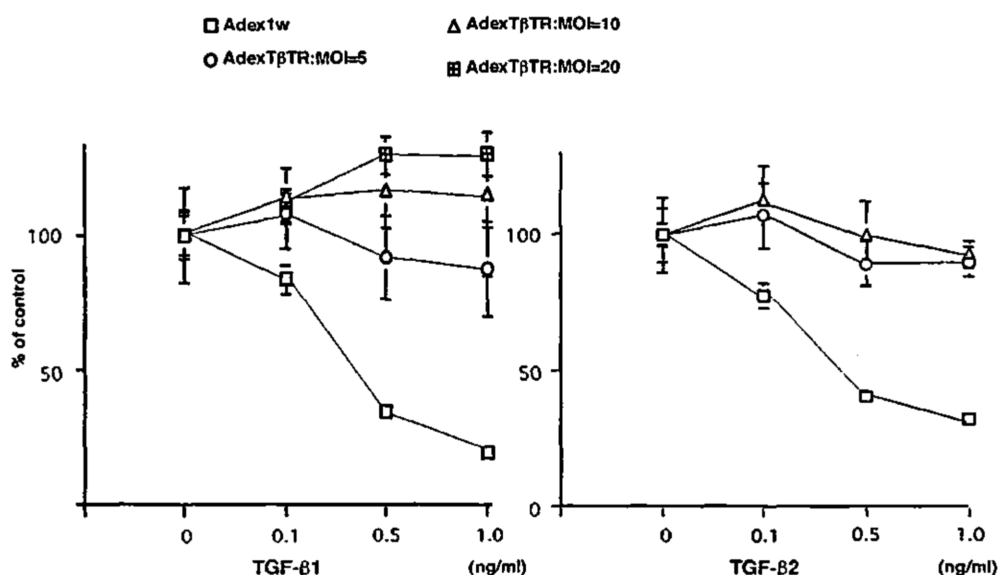

Fig. 3 Complete abolition of TGF-β-induced anti-proliferative effects in NHEK, Exponentially growing NHEK were exposed to *AdexTβTR* and Adex1w at various MOI. After a 24 h incubation, cells were incubated with the indicated concentrations of TGF-β1 or TGF-β2. BrdU was added to each well for 2 h, and its incorporation was measured by ELISA. The data are expressed as the mean of four determinations ±S.D. Three other independent experiments gave similar results.

CMV, and incubated for 24 h. The next day, the medium was changed to MCDB153 basal medium and 10^{-6} M $1\alpha,25(OH)_2D_3$ was added to the medium. Cell lysates were collected at 6, 12, 24 and 48 h; then, the luciferase activity was measured. The TGF-β/Smad-signal-responsive element activity was not altered in the early phase (at 6 and 12 h); however, it increased 2-fold at 24 h and 4-fold at 48 h. This enhancement was completely blocked by pre-treatment with *AdexTβTR*. These results indicate that $1\alpha,25(OH)_2D_3$ enhanced biologically active TGF-β production in NHEK after 24 h (Fig. 4).

3.5. $1\alpha,25(OH)_2D_3$ suppressed the S-phase cell cycle in early-phase growth inhibition

To compare the growth inhibition of $1\alpha,25(OH)_2D_3$ and TGF-β, we investigated the cell cycle after the addition of $1\alpha,25(OH)_2D_3$ and TGF-β. $1\alpha,25(OH)_2D_3$ decreased S-phase cells rapidly from 28.2 to 22.17% at 6 h and to 13.81% at 12 h. TGF-β also decreased S-phase cells from 28.2 to 25.71% at 6 h and to 16.14% at 12 h. Both increased G0/G1 cells from 54.14 to 70% at 12 h. $1\alpha,25(OH)_2D_3$ and TGF-β both induced G0/G1 arrest in NHEK (Fig. 5A). Next we examined the effect of *AdexTβTR* on the cell cycle after $1\alpha,25(OH)_2D_3$ treatment. NHEK were transfected with *AdexTβTR* or *AdexLacZ* (MOI = 5). After 24 h, $1\alpha,25(OH)_2D_3$ was added to the medium and cell cycle analysis was performed. S-phase cells decreased from 26.94 to 18.94% at 6 h and to 11.34% at 12 h in *AdexTβTR*-infected cells. They decreased from 26.4 to 20.2% at 6 h and to 11.83% at 12 h in *AdexLacZ*-infected cells. There was no significant difference about S-phase cells between *AdexTβTR*- and *AdexLacZ*-treated cells (Fig. 5B).

3.6. Growth inhibition by $1\alpha,25(OH)_2D_3$ is not altered in TGF-β-resistant NHEK

NHEK were seeded in 24-well plates at a density of 1 × 10^4 cells/well, and infected with *AdexTβTR* or *AdexLacZ* (MOI = 5). After 24 h, 10^{-6} M $1\alpha,25(OH)_2D_3$ was added to the wells. Six, 12, or 24 h later, the medium was changed to thymidine-free MCDB153 containing 20 mM BrdU, and the cells were incubated for 2 h. BrdU incorporation was measured by ELISA. The addition of 10^{-6} M of $1\alpha,25(OH)_2D_3$ to NHEK infected at an MOI of 5 with *AdexTβTR* and *AdexLacZ* reduced DNA synthesis to 59.3 and 62.2% at 6 h, 24.1 and 31.8% at 12 h, and 25 and 28% at 24 h, respectively. No significant differences were found between *AdexTβTR*- and *AdexLacZ*-infected NHEK (Fig. 6A). Next, we studied the effect of *AdexTβTR* and *AdexLacZ* on clonogenic growth when treated with $1\alpha,25(OH)_2D_3$. NHEK were seeded at a density of 2 × 10^4 cells/well in 6-well plates. The next day, the cells were infected with *AdexTβTR* or *AdexLacZ* at an MOI of 5. After 24 h, 10^{-7} M $1\alpha,25(OH)_2D_3$ was added to the well and incubated for several days. Cell numbers were counted at the indicated times using a Coulter Counter. 10^{-7} M $1\alpha,25(OH)_2D_3$ decreased cell num-

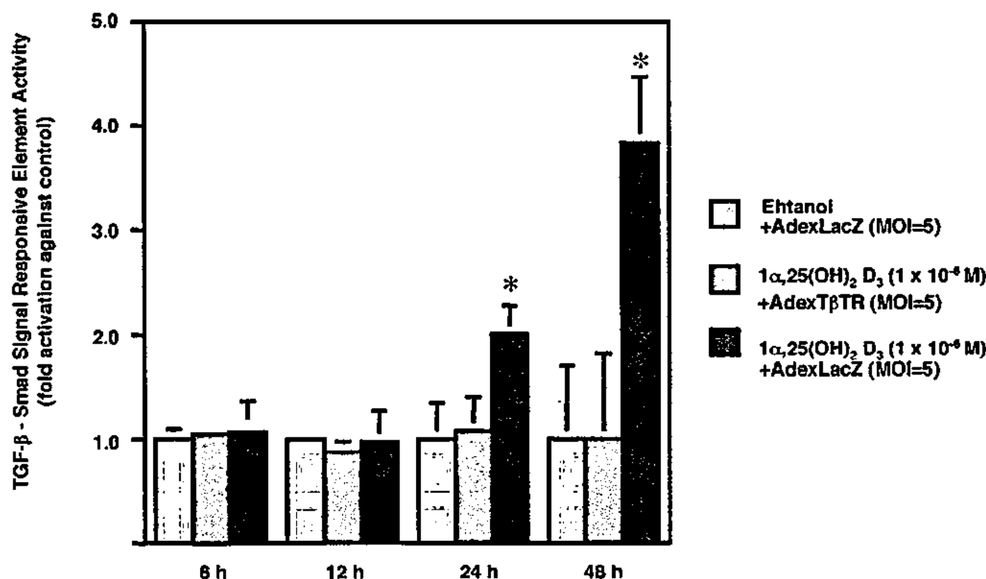

Fig. 4 Effect of $1\alpha,25(OH)_2D_3$ on the induction of TGF-β/Smad-responsive element activity in NHEK. NHEK were infected by *AdexTβTR* or *Adex1w* followed by co-transfection with *p3TP-lux* and *pRL-CMV*, and incubated for 24 h. The next day, the medium was changed to MCDB153 basal medium and 1 × 10^{-6} M $1\alpha,25(OH)_2D_3$ was added to the medium. Cell lysates were collected at 6, 12, 24 and 48 h; then, the luciferase activity was measured. $^*P < 0.01$.

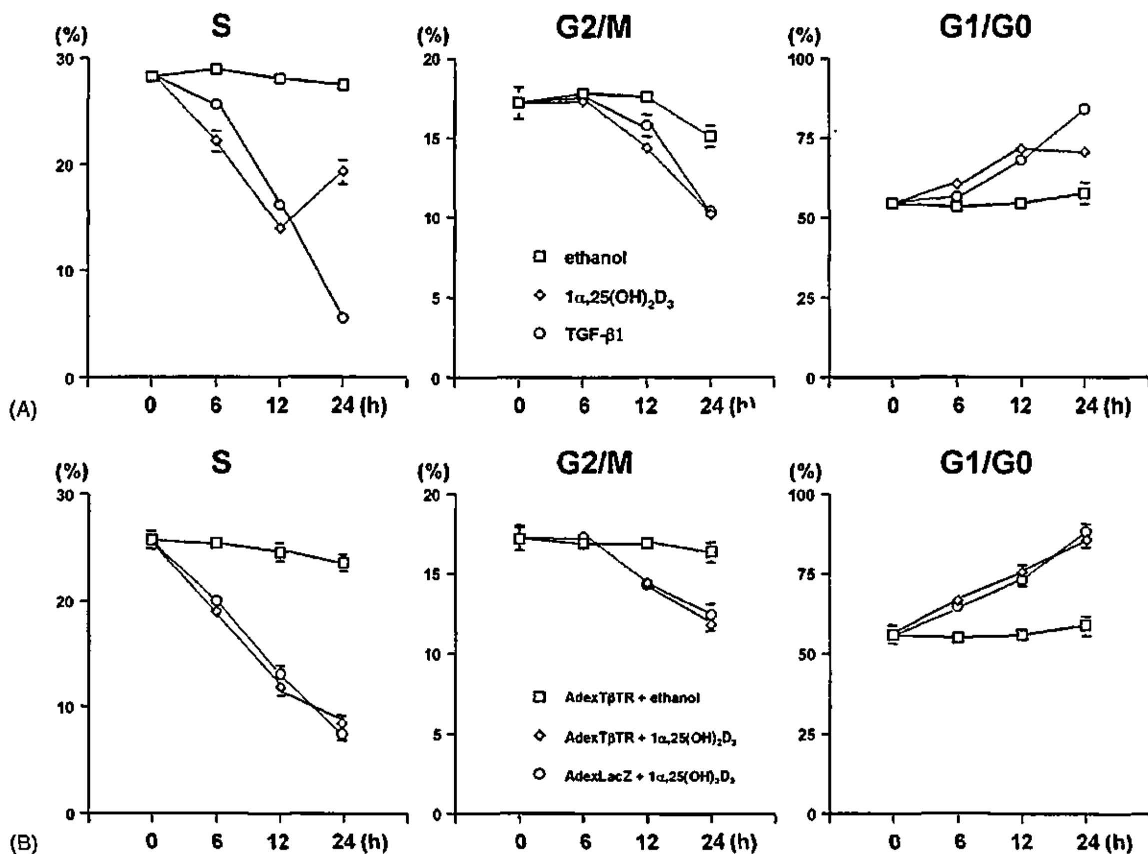

Fig. 5 Cell-cycle analysis, (A) NHEK in the exponential growth phase were treated with $1\alpha,25(OH)_2D_3$ (1×10^{-6} M)and TGF-β (1 ng/ml). Then, the cell cycle was determined using a CycleTEST™ PLUS DNA kit, (B) NHEK were infected with *AdexLacZ* or *AdexTβTR* (MOI = 5). After 24 h, $1\alpha,25(OH)_2D_3$ (1×10^{-6} M) were added to the medium, then the cell cycle was measured.

bers to 70% compared to the control (ethanol addition), although there was no difference between *AdexTβTR*- and *AdexLacZ*-treated cells (Fig. 6B).

4. Discussion

Normal human epidermal keratinocytes are the main component cells of the epidermis, and their growth is regulated by both positive and negative mediators [26,27]. A large number of cytokines and growth factors are known to promote NHEK growth, whereas few negative mediators are known. Several reports have shown that TGF-β, active vitamin D_3 analogue, interferon-γ, ceramide, and apoptosis-signal-regulating kinase 1 inhibit NHEK growth and promote differentiation [7,16,28,29]. Of these cytokines and growth factors, the most important growth inhibitors of NHEK are active vitamin D_3 analogues and TGF-β.

$1\alpha,25(OH)_2D_3$ is the active form of vitamin D_3. Most of the biological actions of active vitamin D_3 analogue are now thought to be mediated through the vitamin D receptor (VDR). The binding of $1\alpha,25(OH)_2D_3$ to the VDR activates target gene expression at the transcription level, with subsequent regulation of physiological events, such as cellular differentiation and proliferation [2–5]. $1\alpha,25(OH)_2D_3$ and other vitamin D_3 analogues have been shown to decrease NHEK proliferation [6,7,30].

TGF-β is a potent growth inhibitor of many epithelial cell types in vitro. The cell membrane receptors for TGF-β are activated by ligand binding, and phosphorylate and activate certain members of the Smad protein family. Extensive studies have examined the molecular mechanism underlying the interplay between TGF-β and vitamin D_3. Through the TGF-β pathway, Smad3, a downstream component of TGF-β signaling, potentiates the ligand-induced transactivation of the VDR [31]. Recently, it was reported that Smad7, one of the inhibitory Smads, inhibits formation of the VDR-Smad3 complex, and negatively regulates VDR transactivation function [32]. On the contrary, Wu et al. found two direct repeat vitamin D response elements in the human TGF-β2 promoter, and found that heterodimers of the VDR and RXRα bind to sequences in the promoter of the TGF-β2 gene [33]. They also demonstrated that vitamin D_3

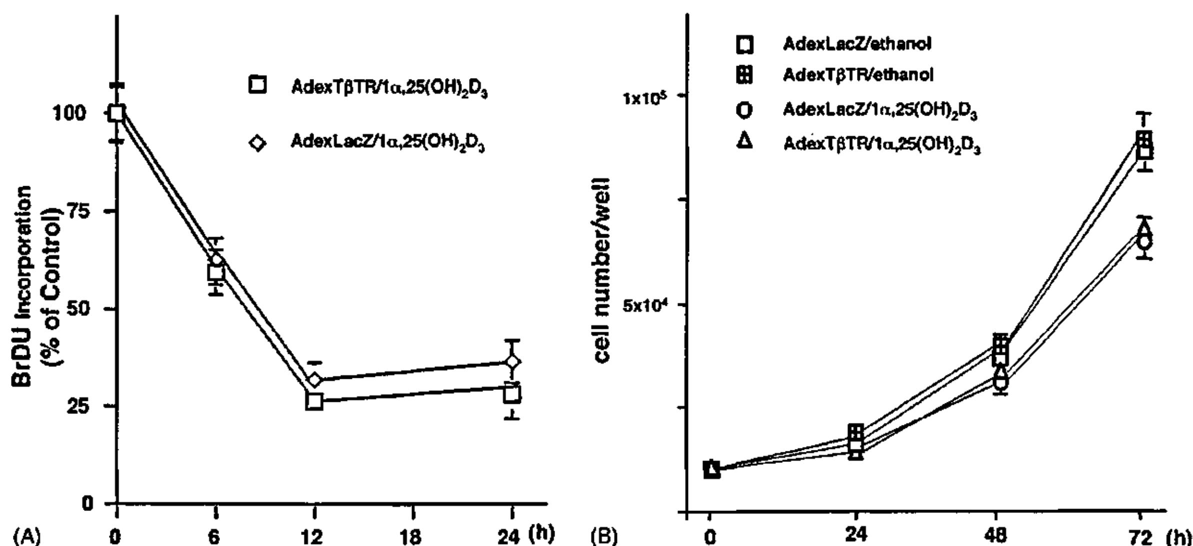

Fig. 6 The effect of $1\alpha,25(OH)_2D_3$ on the growth in *AdexTβTR*-infected NHEK, (A) Exponentially growing NHEK were exposed to *AdexTβTR* or *AdexLacZ* at an MOI of 5. After a 24 h incubation, cells were incubated with 1×10^{-6} M $1\alpha,25(OH)_2D_3$ for the indicated periods. BrdU was added to each well for 2 h, and its incorporation was measured by ELISA. The data are expressed as the mean of four determinations ±S.D. Three other independent experiments gave similar results, (B) The effect of $1\alpha,25(OH)_2D_3$ on subconfluent keratinocytes. Human epidermal keratinocytes were seeded at a density of 2×10^4 cells/well in 6-well plates. The next day, the cells were infected with *AdexTβTR* or *AdexLacZ* at an MOI of 5. After 24 h, 1×10^{-7} M $1\alpha,25(OH)_2D_3$ was added to the wells and incubated for several days. Cell numbers were counted at the indicated times using a Coulter Counter.

upregulates TGF-β type II receptor mRNA and protein levels [34]. TGF-β is thought to be involved in the growth inhibition by vitamin D_3. Kim et al. reported that $1,25(OH)_2D_3$ and TGF-β synergistically decreased NHEK proliferation, and that 10^{-6} M $1,25(OH)_2D_3$ increased TGF-β1 mRNA within 2 h [21]. They also reported that antibodies neutralizing TGF-β blocked the anti-proliferative activity of vitamin D3 by more than 50%. Hagen et al. showed that $1\alpha,25(OH)_2D_3$ inhibited NHEK growth by increasing TGF-β2 release, and that this anti-proliferative effect was partially blocked by the addition of antibodies neutralizing TGF-β [20]. It has been reported that $1,25(OH)_2D_3$ enhances TGF-β1 expression in renal proximal tubular cells, and the expression of TGF-β1 and latent TGF-β binding protein in cultured breast carcinoma cells, and that $1\alpha,25(OH)_2D_3$ increases TGF-β2 and type I and type II receptor expression in human bone cells [35–37]. Therefore, we investigated whether TGF-β is involved in the growth inhibition by $1\alpha,25(OH)_2D_3$ in NHEK. In our experiments, $1\alpha,25(OH)_2D_3$ upregulated TGF-β1 and TGF-β2 mRNA expression 2-fold at 24 h, and 3-fold at 6 h (Fig. 1). Biologically active TGF-β was enhanced 2-fold after 24 h, and 4-fold after 48 h, as assessed by the luciferase reporter assay using the TGF-β/Smad-responsive element (Fig. 4). Combining these findings, $1\alpha,25(OH)_2D_3$ actually upregulates TGF-β synthesis, but it is still controversial how TGF-β is involved in $1\alpha,25(OH)_2D_3$-mediated growth inhibition, because it is difficult to block endogenous TGF-β activity in NHEK completely using neutralizing antibodies.

To overcome this problem, we used a replication-deficient adenovirus vector system, which is a highly effective means of expressing foreign genes in NHEK [38]. We constructed an adenovirus vector expressing a truncated serine/threonine kinase domain of the TGF-β type II receptor with a dominant negative effect (*AdexTβTR*) [19]. In NHEK, *AdexTβTR* blocked TGF-β signal transduction and abolished both endogenous and exogenous TGF-β signaling completely (Fig. 3). Using this *AdexTβTR*, we analyzed whether TGF-β is involved in the growth inhibition by $1\alpha,25(OH)_2D_3$. If TGF-β is involved in a large part of the growth inhibition by $1\alpha,25(OH)_2D_3$, *AdexTβTR* should rescue NHEK from the $1\alpha,25(OH)_2D_3$-mediated growth inhibition. We found no difference in growth inhibition by $1\alpha,25(OH)_2D_3$ between cells infected with *AdexTβTR* and *AdexLacZ* (Fig. 6A). Moreover, we also detected no difference in cell number between these vectors (Fig. 6B). These findings indicate that TGF-β is not involved in the growth inhibition by $1\alpha,25(OH)_2D_3$ under our culture conditions.

Furthermore, we analyzed the biological effect of $1\alpha,25(OH)_2D_3$ on NHEK cell cycle kinetics. $1,25(OH)_2D_3$ inhibits the progression from G1/G0 to S-phase in NHEK and causes dephosphorylation of the retinoblastoma gene product, resulting in growth arrest in G1/G0. The cell number in S phase

decreases at as early as 3 h, and is minimal at 18 h [6,39]. Verlinden et al. reported that in breast cancer cell line MCF-7 cells, $1,25(OH)_2D_3$ inhibited cell proliferation through G1 arrest and decreased the cyclin D1 transcription level [40]. They suggested that the greatest effect of $1,25(OH)_2D_3$ on cell proliferation is likely due to a TGF-β-independent mechanism of action. In NHEK, $1\alpha,25(OH)_2D_3$ decreased S-phase cells rapidly and induced G0/G1 arrest, while TGF-β also decreased S-phase cells after 6 h (Fig. 5A). This inhibitory effect of $1\alpha,25(OH)_2D_3$ on NHEK seems to be the same as that of TGF-β. Furthermore, *AdexTβTR* did not alter the cell cycle distribution by $1\alpha,25(OH)_2D_3$ (Fig. 5B). In a growth assay, the addition of 10^{-6} M $1\alpha,25(OH)_2D_3$ decreased DNA synthesis to 50% at 6 h, and the lowest level occurred at 12 h (Fig. 6A). Although, $1\alpha,25(OH)_2D_3$ upregulated TGF-β1 mRNA from 3 to 24 h and TGF-β2 mRNA from 3 to 6 h after stimulation (Fig. 1), biologically active TGF-β was upregulated after 24 h (Fig. 4). Combining these findings, the early phase growth inhibition of NHEK by $1\alpha,25(OH)_2D_3$ is likely due to a TGF-β-independent mechanism, and is not mediated by the enhancement of TGF-β, although $1\alpha,25(OH)_2D_3$ upregulated TGF-β production.

In conclusion, using an adenovirus vector that expresses a dominant negative TGF-β type II receptor, we clearly demonstrated that TGF-β is not involved in the early phase growth inhibition of human keratinocytes by $1\alpha,25(OH)_2D_3$. These findings contribute to knowledge of the mechanism and biological function of TGF-β and vitamin D3.

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