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# Regulation of RANTES and IL-8 production in normal human dermal fibroblasts by active vitamin $D_3$ (tacalcitol)

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**1** The production of chemokines, RANTES and IL-8 in cultured human dermal fibroblasts and the effects of tacalcitol (1α,24(R)-dihydroxyvitamin $D_3$) were studied using an enzyme-linked immunosorbent assay.

**2** In the unstimulated condition, RANTES and IL-8 were at a trace level in the culture supernatant. On stimulation with TNF-α alone for 24 h, RANTES and IL-8 production were induced. Tacalcitol suppressed RANTES and IL-8 production dose-dependently at concentrations between $10^{-12}$ M and $10^{-7}$ M.

**3** When the cells were treated with TNF-α and IFN-γ in combination, RANTES production was enhanced, but IL-8 production was not changed, compared to TNF-α-treated cells. Tacalcitol decreased IL-8 production dose-dependently as observed in the TNF-α-treated cells. On the other hand, RANTES production was enhanced by $10^{-11}$ M and $10^{-10}$ M of tacalcitol, and dose-dependently suppressed by tacalcitol concentrations higher than $10^{-9}$ M.

**4** Active vitamin $D_3$ compounds, betamethasone valerate and cyclosporin A were compared with respect to their effects on chemokine production. Three active vitamin $D_3$ compounds, tacalcitol, 1α,25-dihydroxyvitamin $D_3$ and MC903 (calcipotriol), inhibited the production of RANTES and IL-8, with very similar potencies. Betamethasone valerate also inhibited these chemokine productions, but with greater potency than active vitamin $D_3$ compounds. Cyclosporin A significantly stimulated RANTES production at $10^{-6}$ M and IL-8 production at $10^{-7}$ M and $10^{-6}$ M.

**5** The results of this study suggest that active vitamin $D_3$ compounds exert some beneficial effects in the treatment of inflammatory skin diseases via regulation of the production of chemokines by dermal fibroblasts.

**Keywords:** RANTES; IL-8; fibroblast; vitamin $D_3$; tacalcitol

## Introduction

Vitamin $D_3$ is synthesized in the skin or derived from nutritional sources, and metabolized first in the liver to 25-hydroxyvitamin $D_3$ and then in the kidney to the hormonally active form, 1α,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$). Active vitamin $D_3$ and related compounds have been shown to be effective in the treatment of psoriasis and other hyperkeratotic skin disorders, as confirmed by a number of trials (Morimoto *et al*., 1986; Kato *et al*., 1986; Gerristen *et al*., 1994; Kragballe, 1989; Cunliffe *et al*., 1992). The mechanism of the therapeutic efficacy of active vitamin $D_3$ is still not fully understood and has been the focus of many investigations, indicating that at least part of the effects can be explained by the regulatory actions on epidermal cell proliferation and differentiation (Matsumoto *et al*., 1990; Kobayashi *et al*., 1990).

Tacalcitol (1α,24(R)-dihydroxyvitamin $D_3$), a synthetic analogue of vitamin $D_3$ is topically applied as an anti-psoriatic drug in an ointment-preparation. Recently, we demonstrated an anti-inflammatory effect of tacalcitol on phorbol ester-induced acute skin reaction in mice (Sato *et al*., 1996). Clinically, tacalcitol ointment reduced the numbers of infiltrating neutrophils, T cells and monocytes in patients' skin (Gerristen *et al*., 1994). These results suggest that the active vitamin $D_3$ compounds have some anti-inflammatory action on cutaneous inflammation, *via* suppression of leukocyte infiltration into cutaneous lesions.

Chemokines are a family of small cytokines (ca.70 residues) produced by a variety of cell types that are able to induce chemotaxis and activation of leukocytes, and are considered to be involved in the pathogenesis of inflammation. RANTES (regulated on activation, normal T expressed and secreted) is a potent chemokine for eosinophils, T cells and monocytes, and plays an important role in recruiting leukocytes to inflammatory sites (Kameyoshi *et al*., 1992; Schall *et al*., 1990). IL-8 is also a chemokine specific for neutrophils and T cells, and has been shown to be overexpressed in psoriatic lesion (Gillitzer *et al*., 1996; Kulke *et al*., 1996).

In this study, we focused on RANTES and IL-8 production in cultured normal human dermal fibroblasts (NHDF). By using an ELISA system, we assayed the effect of tacalcitol on these chemokine productions. Furthermore, we compared active vitamin $D_3$ compounds, a synthetic steroid and an immunosuppressant with respect to their effects on chemokine production.

## Methods

### *Reagents*

Recombinant human TNF-α (specific activity = $2-5\times10^4$ U $\mu g^{-1}$), IL-1β ($1-2\times10^5$ U $\mu g^{-1}$), IFN-γ ($1\times10^4$ U $\mu g^{-1}$) and IL-4 ($0.5-2\times10^4$ U $\mu g^{-1}$) were obtained from R&D systems (Minneapolis, MN, U.S.A.). Betamethasone valerate and cyclosporin A were obtained from WAKO Purechemicals (Tokyo, Japan). Tacalcitol, $1,25(OH)_2D_3$ and

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MC903 were synthesized at TEIJIN Institute for Bio-Medical Research (Tokyo, Japan).

### *Cells and medium*

Primary normal human dermal fibroblasts (NHDF) derived from neonatal donors and FGM medium (modified MCDB202 containing 2% fetal bovine serum, fibroblast growth factor, insulin, and antibiotics) were purchased from Clonetics (CA, U.S.A.).

### *Culture condition*

The culture was maintained in an incubator at 37°C under a 5% $CO_2$ atmosphere. The culture medium was changed every 3 days and the cells were passaged at subconfluence in a split ratio of about 1:3–4. Third to fourth passaged cells were plated on a 48 well-culture plate with $2-4\times10^4$ cells per well in 0.5 ml medium and cultured for 2 days. The cells were incubated in the medium containing stimulants and/or testing compounds. After 24 or 48 h, the culture supernatants were collected and stored at −80°C until use.

### *Measurement of chemokine*

Concentrations of chemokines were determined by a sandwich-type enzyme-linked immunosorbent assay using a human RANTES ELISA system and a human IL-8 ELISA system (Amersham, U.K.). The sensitivities of these assays were 2.5 and 5 pg/ml, respectively.

### *Statistical analysis*

Results are expressed herein as the means + standard deviation (s.d.). Statistical analysis was performed by Dunnett's two-tailed test, by using the software 'super ANOVA' (Abacus Concept, Inc., CA, U.S.A.) on a Macintosh computer. A $P$ value of less than 0.05 was considered to be significant.

## Results

### *RANTES production in cultured NHDF*

To examine the induction of RANTES production in NHDF *in vitro*, the cells were treated with various cytokines for 24 h, and the chemokine concentrations in the culture supernatants were determined by ELISA. As shown in Figure 1a, RANTES was at a trace level in the unstimulated condition. On stimulation with TNF-α alone, RANTES production increased significantly (898 pg/ml). When the cells were treated with TNF-α and IFN-γ in combination, RANTES production was enhanced by 4.3 fold as compared to TNF-α-treated cells, while IFN-γ alone had no inductive effect. IL-4 suppressed the RANTES production induced by TNF-α and IFN-γ, while enhancing the RANTES production induced by TNF-α. IL-1β alone also induced chemokine production, but with limited potency as compared to TNF-α.

### *IL-8 production in cultured NHDF*

The IL-8 production by NHDF was assessed by using the same culture supernatants as described above (Figure 1b). IL-8 production was induced by TNF-α alone or IL-1β alone, or a combination of the two (30.5 ng $ml^{-1}$, 49.9 ng $ml^{-1}$ and 64.5 ng $ml^{-1}$, respectively). IFN-γ showed neither induction by itself, nor synergism with TNF-α or IL-1β in inducing IL-8 production. IL-4 weakly suppressed IL-8 production.

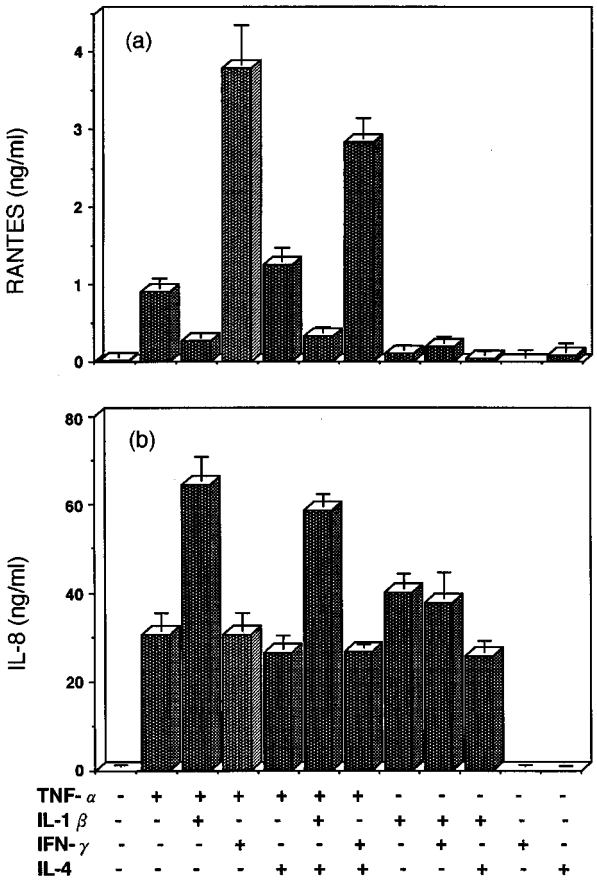


**Figure 1** (a) Induction of RANTES and (b) IL-8 production in normal human dermal fibroblasts (NHDF). NHDF cultures were treated with 10 ng $ml^{-1}$ TNF-α, 5 ng $ml^{-1}$ IL-1β, 10 ng $ml^{-1}$ IFN-γ, 10 ng $ml^{-1}$ IL-4, alone or in combination for 24 h. The chemokine concentrations in the supernatants were determined by ELISA. Results are expressed as mean + s.d. $n=4$.

### *Time-course of RANTES production*

For the time-course study, we examined the production of RANTES and IL-8 in cultured NHDF at various times after the addition of TNF-α and/or IFN-γ. Figure 2a shows the RANTES production. In the unstimulated and IFN-γ-stimulated conditions, RANTES was at a trace level throughout the experiment. In TNF-α-treated cells, the rate of RANTES production increased steadily, even after 96 h. On the other hand, in the cells treated with TNF-α and IFN-γ in combination, the production rate increased within 24 h and remained stable from 24 to 96 h, then decreased up to 120 h. Thus, TNF-α is essential for RANTES production, and IFN-γ enhances the responsiveness of cells to the action of TNF-α.

### *Time-course of IL-8 production*

As shown in Figure 2b, IL-8 was not detectable in the unstimulated and IFN-γ-stimulated conditions. The IL-8 concentration increased steadily up to 120 h. There was no difference between the cells treated with TNF-α alone and those treated with TNF-α and IFN-γ in combination. In

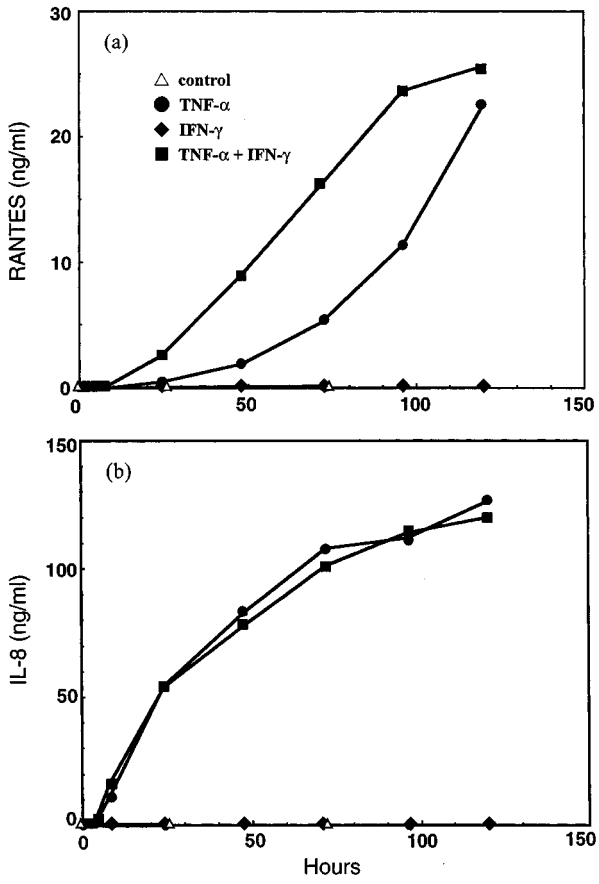


**Figure 2** Time courses of RANTES (a) and IL-8 (b) productions induced by TNF-α and/or IFN-γ in NHDF. NHDF cultures were treated with 10 ng $ml^{-1}$ TNF-α and 10 ng $ml^{-1}$ IFN-γ, alone or in combination for 1, 2, 4, 8, 24, 72, 96 and 120 h. The chemokine concentrations in the supernatants were determined by ELISA. Results are expressed as mean. $n=2$.

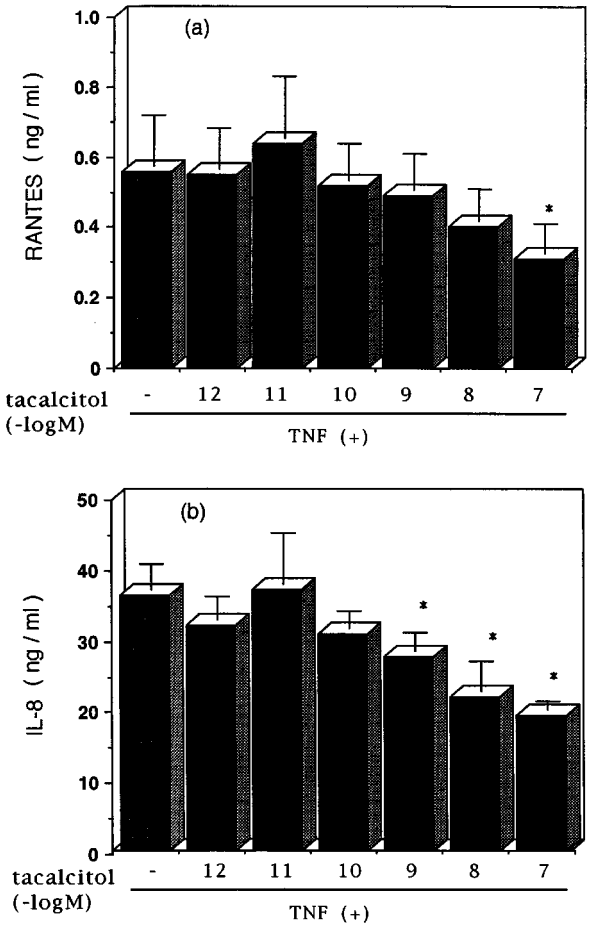


**Figure 3** Effect of tacalcitol on RANTES (a) and IL-8 (b) production induced by TNF-α in NHDF. NHDF cultures were treated with 10 ng $ml^{-1}$ TNF-α in the presence of tacalcitol ($10^{-12}$–$10^{-7}$ M) for 24 h. The chemokine concentrations in the supernatants were determined by ELISA. Results are expressed as mean + s.d. $n=6$. *Indicates a statistically significant difference with $P<0.05$, compared with the control group by Dunnett's two-tailed test.

contrast to RANTES production, the onset of IL-8 production was faster and the rate peaked between 8 and 24 h, thereafter decreasing gradually.

### *Effects of tacalcitol on RANTES and IL-8 productions*

We have evaluated the effects of tacalcitol on RANTES and IL-8 production in NHDF. Cells were treated with tacalcitol over the concentration range from $10^{-12}$ M to $10^{-7}$ M for 24 h in the presence of TNF-α. As shown in Figure 3, the productions of RANTES and IL-8 were inhibited by tacalcitol in a dose-dependent fashion. RANTES and IL-8 productions were suppressed by $10^{-7}$ M of tacalcitol to 55% and 50% of the control level, respectively. When cells were treated with TNF-α and IFN-γ in combination, the effect of tacalcitol on RANTES production was biphasic as shown in Figure 4a; RANTES production was enhanced by $10^{-11}$ M and $10^{-10}$ M of tacalcitol and suppressed by the higher concentration of tacalcitol in a dose-dependent fashion. The RANTES concentrations at $10^{-10}$ M and $10^{-7}$ M of tacalcitol were 134% and 62% of the control level, respectively.

On the other hand, in the case of IL-8, dose-dependent inhibition by tacalcitol was observed (Figure 4b). At $10^{-7}$ M of tacalcitol, IL-8 secretion was suppressed to 53% of the control level. In the absence of cytokine stimulation, tacalcitol itself did not induce RANTES or IL-8 production by NHDF.

### *Comparisons among active vitamin $D_3$ compounds, betamethasone valerate and cyclosporin A*

Some anti-psoriatic drugs were compared in terms of their suppressive effects on chemokine production. Cells were treated with tacalcitol, $1,25(OH)_2D_3$, MC-903 (calcipotriol), betamethasone valerate and cyclosporin A for 48 h in the presence of TNF-α and IFN-γ. As shown in Figure 5, the productions of RANTES and IL-8 were inhibited by the three active vitamin $D_3$ compounds in a dose-dependent fashion, with the potencies being essentially the same. Betamethasone valerate also inhibited these chemokine productions; the effect on RANTES production was stronger than that for active vitamin $D_3$ compounds, while the effect on IL-8 production was apparently comparable. On the contrary, cyclosporin A significantly stimulated RANTES production at $10^{-6}$ M and IL-8 production at $10^{-7}$ M and $10^{-6}$ M.

## Discussion

RANTES has been reported to be a chemokine specific for memory T cells, eosinophils and monocytes, but not for neutrophils (Kameyoshi *et al*., 1992). The role of RANTES in inflammatory skin disease is unclear, though its biological

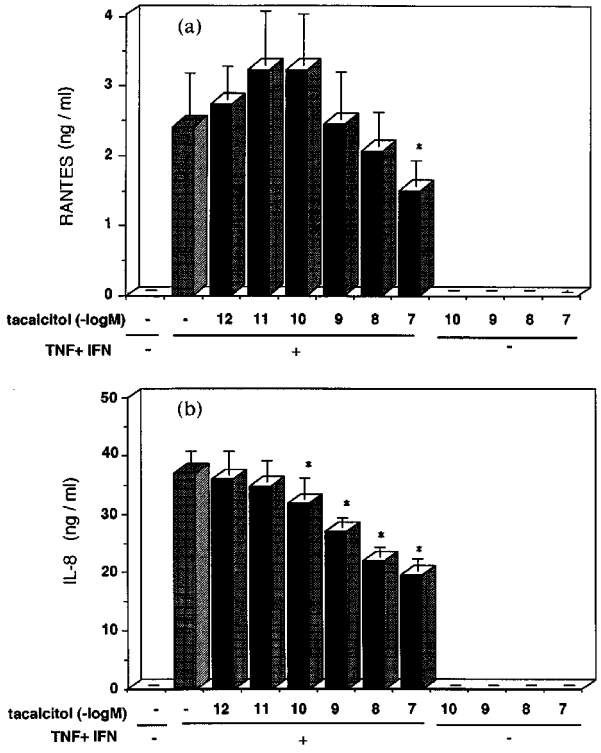


**Figure 4** Effect of tacalcitol on RANTES (a) and IL-8 (b) production induced by TNF-$\alpha$ and IFN-$\gamma$ in NHDF. NHDF cultures were treated with 10 ng $ml^{-1}$ TNF-$\alpha$ and 10 ng $ml^{-1}$ IFN-$\gamma$ in the presence of tacalcitol ($10^{-12}$–$10^{-7}$ M) for 24 h. The chemokine concentrations in the supernatants were determined by ELISA. Results are expressed as mean + s.d. $n=6$. *Indicates a statistically significant difference with $P<0.05$, compared with the control group by Dunnett's two-tailed test.

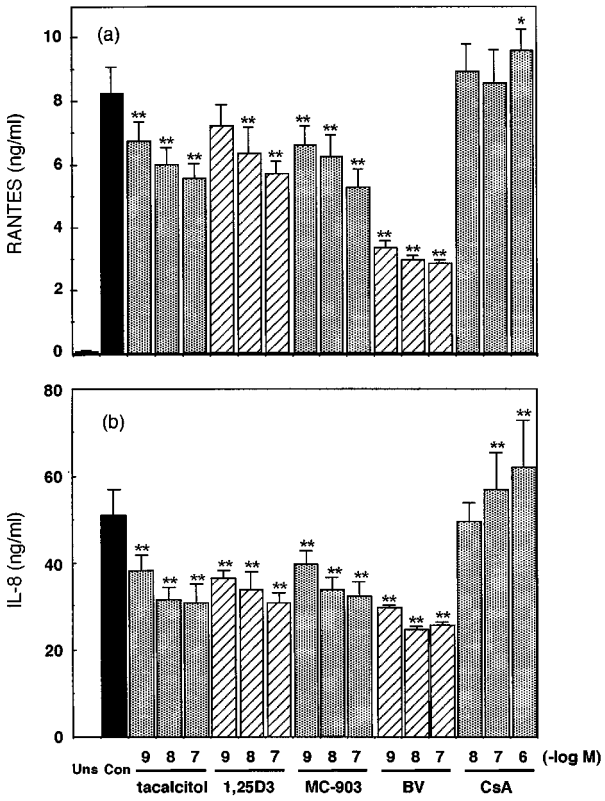


**Figure 5** Effects of active vitamin $D_3$ compounds, betamethasone valerate and cyclosporin A on RANTES (a) and IL-8 (b) production induced by TNF-$\alpha$ and IFN-$\gamma$ in NHDF. NHDF cultures were treated with 5 ng $ml^{-1}$ TNF-$\alpha$ and 1 ng $ml^{-1}$ IFN-$\gamma$ for 48 h, in the presence of tacalcitol ($10^{-9}$–$10^{-7}$ M), $1,25(OH)_2D_3$ ($10^{-9}$–$10^{-7}$ M), MC903 ($10^{-9}$–$10^{-7}$ M), betamethasone valerate ($10^{-9}$–$10^{-7}$ M), or cyclosporin A ($10^{-8}$–$10^{-6}$ M). The chemokine concentrations in the supernatants were determined by ELISA. Results are expressed as the mean + s.d. $n=5$. Uns, unstimulated; Con, control; 1,25D3, $1,25(OH)_2D_3$; BV, betamethasone valerate; CsA, cyclosporin A. *,**Indicates the statistically significant difference with $P<0.05$, $P<0.01$, compared with the control group by Dunnett's two-tailed test.

properties make this chemokine an attractive candidate for the mediator in allergic skin reactions. Meurer *et al.* (1993) reported that direct injection of human RANTES into canine skin induced local accumulation of eosinophils and monocytes. Ying *et al.* (1995) demonstrated that intradermaly applied antigen induced eosinophil, T cell, and macrophage infiltration in atopic subjects, in whom RANTES mRNA expression was observed to parallel T cell infiltration. These reports reveal that RANTES has significant proinflammatory activity in allergic skin reactions.

In this study, we confirmed the production of RANTES by NHDF. The optimal production was achieved by stimulation with TNF-$\alpha$ and IFN-$\gamma$ in combination. IL-4 partially suppressed the RANTES production induced by TNF-$\alpha$ and IFN-$\gamma$ in combination. The enhancement by IFN-$\gamma$ and the suppression by IL-4 are consistent with the results reported for RANTES production observed in various cell types such as epidermal keratinocytes (Fukuoka *et al.*, 1998), synovial fibroblasts (Rathanaswami *et al.*, 1993) and umbilical vein endothelial cells (Marfaing-Koka *et al.*, 1995).

Psoriasis is a chronic inflammatory skin disease characterized by hyperproliferation and defective differentiation of epidermal keratinocytes. The skin lesion of psoriasis typically involves Munro's microabscess, intracorneal or subcorneal vesicles consisting of neutrophil infiltrates, suggesting the importance of neutrophils in the pathogenesis of psoriasis. In this regard, several reports have shown that IL-8, a strong chemotactic mediator for neutrophils and lymphocytes, is produced by the lesional keratinocytes of psoriasis (Gillitzer *et al.*, 1996; Kulke *et al.*, 1996). Although IL-8 transcripts were reported to be absent in the dermal compartment of psoriasis (Gillitzer *et al.*, 1996; Kulke *et al.*, 1996), our results of fibroblast suggest a model system for analysing the cellular and molecular mechanisms underlying the regulation of chemokine production. As shown in Figures 1b and 2b, the inductive activities of TNF-$\alpha$ and IL-1$\beta$ on IL-8 production were additive. Differing from the induction of RANTES, IFN-$\gamma$ showed no enhancement of the induction of IL-8 by TNF-$\alpha$.

We evaluated the effects of tacalcitol on RANTES and IL-8 productions by cytokine-stimulated NHDFs. Tacalcitol exhibited a significant inhibition in these two chemokine productions, as shown in Figures 3 and 4. We have also reported that tacalcitol suppressed the production of RANTES and IL-8 in human epidermal keretinocytes (Fukuoka *et al.*, 1998). In addition, Larsen *et al.* (1991) showed that $1,25(OH)_2D_3$ suppressed IL-8 production in cultured epidermal keratinocytes. Taken together, these observations suggest that active vitamin $D_3$ compounds such as tacalcitol may inhibit the migration of granulocytes and lymphocytes into skin lesions, by reducing the chemokines which are produced by dermal fibroblasts and keratinocytes. The inhibition of IL-8 production by tacalcitol in keratinocytes partly explains the clinical efficacy of active vitamin $D_3$ as an anti-psoriatic drug. The inhibition of RANTES production by

tacalcitol suggests the potential of active vitamin $D_3$ compounds as a therapy for allergic skin diseases. In fact, Katayama *et al*. (1996) reported that topical tacalcitol downregulated the hapten-specific cutaneous reactions (immediate and delayed-onset) which were induced in mice passively sensitized with monoclonal anti-DNP IgE.

Tacalcitol, $1,25(OH)_2D_3$, MC-903 (calcipotriol) (Kragballe, 1989; Cunliffe *et al*., 1992; Calverley, 1987), betamethasone valerate and cyclosporin A were compared in terms of their suppressive effects on chemokine production. The productions of RANTES and IL-8 were inhibited by the three active vitamin $D_3$ compounds and betamethasone valerate. Although $1,25(OH)_2D_3$ (Clements *et al*., 1983) and betamethasone valerate (Hein *et al*., 1994) has also reported to inhibit the growth of NHDFs, the cells used were at confluent stage when treated with the cytokines and compounds in our assay. In addition, it has been reported that $1,25(OH)_2D_3$ up to $10^{-6}$ M did not affect the viability of NHDFs as assessed by trypan blue dye exclusion test. Thus, the growth-inhibitory and cytotoxic action to NHDFs seems less important for the inhibitory effect on chemokine productions demonstrated.

Since the specific receptor for active vitamin $D_3$ (VDR), as well as the glucocorticoid receptor, belongs to the nuclear receptor superfamily (Evans, 1988), the two hormones may act through a similar pathway. Glucocorticoids have been shown to exert their potent anti-inflammatory action *via* the inhibition of gene expressions, such as those of inflammatory cytokines and intercellular adhesion molecules (Nelson *et al*., 1993). The inhibition of gene expression by glucocorticoids results from interference with the activity of transcription factors such as AP-1 and NF-$\kappa$B (Cato & Wade, 1996). Since a $\kappa$B site and AP-1 site exist in the region upstream from the RANTES (Nelson *et al*., 1993) and IL-8 genes (Yasumoto *et al*., 1992), the mechanisms by which active vitamin $D_3$ and glucocorticoids inhibit chemokine productions may involve inhibition of the activities of these transcription factors.

In contrast to active vitamin $D_3$ compounds and glucocorticoids, cyclosporin A significantly stimulated RANTES and IL-8 production, as shown in Figure 5. Other reports have shown cyclosporin A (Elder *et al*., 1993; Kaplan *et al*., 1995) and FK-506 (Kaplan *et al*., 1995) to be ineffective in inhibiting cytokine-stimulated IL-8 production in cultured epidermal keratinocytes. Thus, in the treatment of psoriatic skin lesions, active vitamin $D_3$ and glucocorticoids act directly on fibroblasts and keratinocytes, while immunosuppressants such as cyclosporin A and FK-506 act through activated T lymphocytes rather than keratinocytes and fibroblasts.

In conclusion, we demonstrated RANTES and IL-8 productions in cultured NHDF. In addition, tacalcitol was shown to inhibit the production of RANTES and IL-8 at appropriate concentrations. These results suggest that the suppression of chemokine production by active vitamin $D_3$ in dermal fibroblasts contributes to its efficacy in the treatment of inflammatory skin diseases.

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