Acta Derm Venereol (Stockh) 1994; Suppl. 186: 52–54

Effect of Calcitriol on Growth, Differentiation, Chemokine mRNA Expression of Cultured Keratinocytes and on Keratinocyte-T Cell Binding

P. de HAAN, S. SAMPAT, E. M. H. van der RAAIJ, I. BRUYNZEEL, D. M. BOORSMA, R. WILLEMZE and T. J. STOOF

Department of Dermatology, Free University Hospital, Amsterdam, The Netherlands

Calcitriol has recently been shown to be effective against psoriasis. However, its mode of action is not exactly known. The present study focused on the influence of calcitriol on growth, differentiation, chemokine mRNA and ICAM-1 mRNA expression of keratinocytes (KC) and on the binding of T-cells to keratinocytes. In vitro studies showed that calcitriol has a strong anti-proliferative effect and induces terminal differentiation. γ-IP-10 and ICAM-1 mRNA were induced by γ-IFN, an induction not influenced by calcitriol. Moreover, the functional expression of ICAM-1 on the KC cell surface as measured by a cell adhesion assay, was not influenced either. IL-8 and huGRO mRNAs were constitutively produced in KC, as was demonstrated after incubation with cycloheximide. Up-regulation of both IL-8 and huGRO mRNA by IL-1α was also not affected by calcitriol. It is concluded that calcitriol has a strong antiproliferative activity and does not interfere with KC responsiveness to γ-IFN and IL-α induced chemokine expression or with the adhesion of T-cells to keratinocytes.

Acta Derm Venereol (Stockh) Suppl. 186: 52–54.

P. de Haan, Dept. of Dermatology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands.

Psoriasis is characterized by hyperproliferation and impaired differentiation of keratinocytes (KC) and the presence of activated T cells and neutrophils in the epidermis (1, 2). KC-leukocyte interactions and chemotactic factors are believed to play a crucial role in the pathogenesis of psoriasis (3, 4).

Recently a superfamily of chemokines with chemotactic properties has been described (5). γ-IP-10, IL-8 and huGRO, members of this superfamily, can be induced in vitro by pro-inflammatory cytokines (γ-IFN, IL-1 and TNF-α) and have been detected in psoriatic scales. Therefore they may be involved in the leukocyte infiltration (6, 7). Recent investigations demonstrated that calcitriol is effective in psoriasis. However, the way in which calcitriol exerts its beneficial effects is not exactly known. Several studies have demonstrated that it causes normalization of epidermal differentiation and suppresses leukocyte infiltration (8). In addition it was shown that calcitriol decreases IL-8 levels in KC (9); the effects on the other chemokines are currently unknown. Moreover, whether or not calcitriol interferes with the binding of T-cells to KC is unknown either.

The present study focuses on the effects of calcitriol on growth, differentiation and chemokine mRNA expression in cultured KC and the binding of T-cells to keratinocytes.

MATERIALS AND METHODS

Culturing conditions

Human foreskin keratinocytes (KC) were cultured in serum-free medium (keratinocyte-SFM, Gibco, Breda, The Netherlands) as described previously (3, 10). All studies were performed under low Ca^{++} (0.1 mM) culture conditions, except for the proliferation study which was done under high Ca^{++} (1.8 mM) conditions.

For proliferation studies, third passage KC (5×10^3 cells/cm^2) were grown for 48 h with or without calcitriol (10^{-7}, 10^{-8} M), kindly provided by Solvay Duphar, Weesp, The Netherlands. The cells were labelled with [^{3}H] thymidine (5 μCi/ml) for 4 h. Incorporation of [^{3}H] thymidine was expressed as the percentage of standard incorporation of controls (11). Experiments were performed in triplicate.

To measure differentiation of KC, the percentage involucrin- positive cells was determined (12). Therefore third passage KC (5×10^3 cells/cm^2) were grown for 96 h with or without calcitriol (2.5×10^{-7} M). After trypsinization, fixation and immunocytochemical staining with an antibody against involucrin (14), the cells were transferred to object slides and counted microscopically (12).

For RNA isolation, subconfluent cultures of KC (3–5 passages) were grown in the presence or absence of calcitriol (final concentration 10^{-7}, 10^{-8}, 10^{-9} M) with either TNF-α (1000 U/ml), IL-1α (1000 U/ml) or γ-IFN (300 U/ml), for the indicated periods of time (see legends to the figures). In some experiments, cycloheximide (CHX, 10 μg/ml) was added.

RNA isolation and Northern blot analysis

Total RNA was isolated from KC using the guanidinium-HCl method (3). RNA samples (10 μg) were fractionated on 1% agarose gels containing 2.2 M formaldehyde and transferred to Nylon Hybond N filters (Amersham, Slough, Bucks, England). The blots were hybridized with random ^{32}P labelled probes specific for huGRO, γ-IP-10, IL-8 and ICAM-1, while cyclophillin or β-actin were used as "housekeeping" genes.

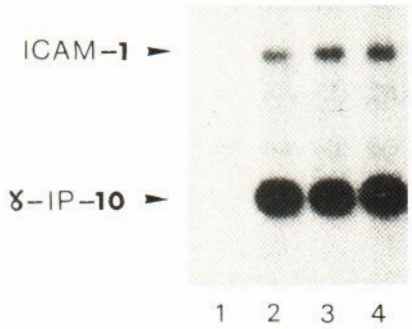

Fig. 1. Effect of calcitriol (D3) on the induction of ICAM-1 and γ-IP10 mRNA in KC. Northern blot analysis of RNA obtained from unstimulated KC (*lane 1*)and from KC stimulated for 3 h with γ-IFN alone (300 units/ml, *lane 2*)and γ-IFN (300 units/ml) in combination with D3 (10^{-7}M, *lane 3*; 10^{-8} M, *lane 4*). Total cellular RNA (10 μg/lane) was probed for ICAM-1 and γ-IP10.

 ISSN 0365-8341

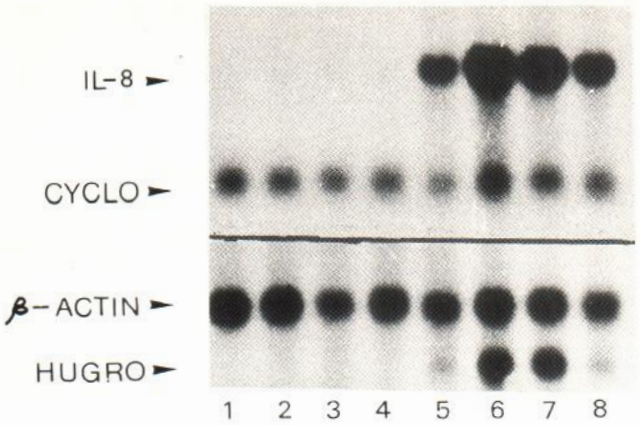

Fig. 2. Effect of Calcitriol (D3) on the induction of IL-8 and huGRO mRNA in KC. Composite figure of Northern blot analysis of RNA obtained from unstimulated KC (*lane 1*) and stimulated (3 h) KC. *Lane 2*: IL-1α (1000 units/ml); *lane 3*: IL-1α (1000 units/ml) and D3 (10^{-7} M); *lane 4*: IL-1α (1000 units/ml) + D3 (10^{-8} M); *lane 5*: cycloheximide (CHX, 5 μg/ml); *lane 6*: IL-1α (1000 units/ml) + CHX (5 μg/ml); *lane 7*: IL-1α + D3 (10^{-7} M) + CHX (5 μg/ml); *lane 8*: CHX (5 μg/ml) + D3 (10^{-7} M). Total cellular RNA (10 μg/lane) was probed for IL-8 and huGRO. Cyclophillin and β-actin were used as reference genes.

Cell adhesion assay

The adhesion of T-cells to the keratinocyte cell-line SVK-14 was measured in the enzyme-linked immuno-cell adhesion assay (10). Subconfluent cultures of SVK-14 cells were treated for 48 h with γ-IFN (300 U/ml) in the presence or absence of calcitriol (final concentration 10^{-7} M). Unstimulated or PMA stimulated (50 ng PMA/ml; 30 min.) normal human T-cells were added to KC and after 1 h of incubation the bound T-cell fraction was quantified spectrophotometrically.

RESULTS

Effect of calcitriol on proliferation and differentiation

[^{3}H]-thymidine incorporation in KC, reflecting cell proliferation, was reduced to 51.8% by 10^{-8} M calcitriol and to 26.8% by 10^{-7} M calcitriol as compared with untreated controls. The percentage involucrin-positive cells indicative of differentiation increased from 17% in control KC (without calcitriol) to 80% in KC grown in the presence of calcitriol.

Effect of Calcitriol on ICAM-1, γ-IP-10, IL-8 and huGRO mRNA expression

Cultured human KC did not produce detectable amounts of mRNAs coding for ICAM-1 and γ-IP-10 respectively (Fig. 1, lane 1). Even in the presence of CHX, there were no dectectable signals indicating that in growing KC, ICAM-1 and γ-IP-10 mRNAs are not constitutively produced (data not shown). KC can be induced to express ICAM-1 and γ-IP-10 mRNA with γ-IFN (Fig. 1, lane 2). Simultaneous addition of calcitriol and γ-IFN had no effect on the expression of ICAM-1 and γ-IP-10 mRNA (Fig. 1, lanes 3, 4). IL-8 and huGRO mRNAs are undetectable in normal KC (Fig. 2, lane 1), but accumulate in the presence of CHX (Fig. 2, lane 5), indicating that IL-8 and huGRO mRNA are constitutively expressed in growing keratinocytes. Addition of calcitriol had no effect on the constitutive synthesis of IL-8 and huGRO mRNAs (Fig. 2, lane 8).

IL-1α did not appear to induce IL-8 and huGRO mRNA to any detectable degree (Fig. 2, lane 2). However, in the presence of CHX, the IL-1α induced up-regulation of IL-8 and huGRO mRNA expression became very obvious (Fig. 2, lane 6). Addition of calcitriol had no effect on this IL-1α-induced IL-8/huGRO mRNA synthesis (Fig. 2, lanes 6, 7).

From a theoretical point of view one might argue that treatment with calcitriol for only 3 h (Figs 1 and 2) is insufficient to have any effect. That prompted us to choose an experimental approach for IL-8 and huGRO (Fig. 3) where KC were pretreated with calcitriol for 0, 5, 24 and 48 h prior to stimulation. From Fig. 3 it can be concluded that KC retain their capacity to produce IL-8 and huGRO mRNA following pretreatment with calcitriol, even after 48 h.

Although the strong growth-inhibiting properties of calcitriol are reflected by an overall down-regulation of mRNA synthesis, including the reference genes cyclophillin and beta-actin, (Fig. 3, lanes 13–16), the IL-8 to cyclophillin and the huGRO to β-actin ratios are not influenced by calcitriol, indicating that calcitriol does not selectively interfere with IL-8 and huGRO mRNA synthesis in KC.

Effect of calcitriol on KC-T-cell adhesion

Non-actived T-cells became minimally bound to unstimulated

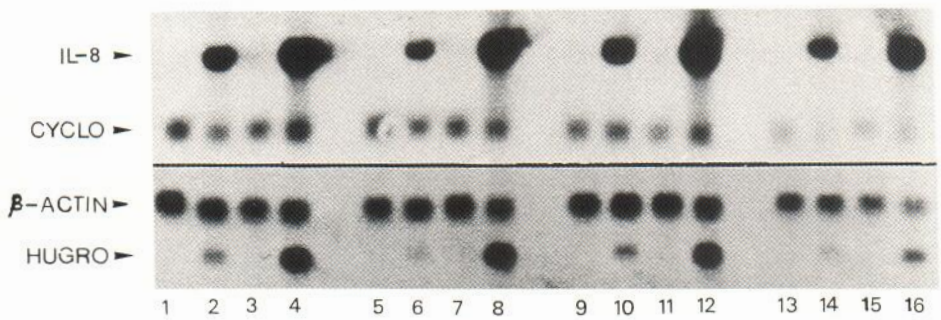

Fig. 3. Long-term effect of Calcitriol (D3) on the induction of IL-8 and huGRO mRNA in KC; a time course study KC were preincubated with D3 (10^{-7} M) for 0 h (*lanes 1–4*), 5 h (*lanes 5–8*), 24 h (*lanes 9–12* and 48 h (*lanes 13–16*) respectively. At the indicated time points following pre-treatment with D3 the KC were incubated for 3 h with: D3 (10^{-7} M), lanes 1, 5, 9, 13; CHX (5 μg/ml) + D3 (10^{-7} M), lanes 2, 6, 10, 14; IL-1α (1000 units/ml) + D3 (10^{-7} M), lanes 3, 7, 11, 15; and CHX + IL-1α + D3, lanes 4, 8, 12, 16. Total cellular RNA (10 μg/lane) was probed for IL-8, huGRO, cyclophilin and β-actin.

SVK-14 cells, in contrast to PMA-activated T-cells. After stimulation of SVK-14 cells by γ-IFN and a two-fold increase in binding of activated T-cells was found. Preincubation with anti-ICAM-1 inhibited the binding of T-cells to KC to 50% (10).

Addition of calcitriol simultaneously with γIFN activation of the SVK-14 cells had no effect on the proportion of adhering T-cells to this keratinocyte cell-line, as compared with calcitriol-untreated controls, which is consistent with our observation that ICAM-1 expression is not influenced by calcitriol.

DISCUSSION

In this study we investigated the effect of calcitriol on normal human KC with respect to growth, differentiation, chemokine mRNA production and T-cell adhesion in order to get an insight into the mode of action underlying its beneficial effects in psoriasis.

Calcitriol appeared to strongly inhibit proliferation and to induce differentiation, probably by increasing intracellular Ca^{++} levels (14). These findings are in agreement with data from the literature (11).

In psoriatic skin, increased amounts of the chemokines γ-IP-10, IL-8 and huGRO, which are chemotactic for T-cells and neutrophils, have been found (4, 7). Of these chemokines, γ-IP-10 and IL-8 proved normal after successful treatment with tar, UV, etretinate, or corticosteroids (4, 12). For calcitriol treatment, no data are available as yet. In this study we demonstrate that in appropriately stimulated KC, calcitriol does not selectively suppress gene transcription of these three chemokines. Though Larsen et al. (9) observed some suppression of IL-8 gene transcription in KC under the influence of calcitriol, we did not detect such an effect.

We also investigated the effect of calcitriol on γ-IFN-induced ICAM-1 expression in KC. Calcitriol did not seem to interfere with this stimulus-induced ICAM-1 mRNA expression at early time points (3 h of incubation). Moreover, calcitriol had no effect on adhesion of activated T-cells to KC monolayers, indicating that the functional expression of ICAM-1 on KC cell surface was not influenced by calcitriol. Matsumoto et al. (15) found a reduction in the number of high affinity EGF-receptors following incubation with calcitriol for 48 h. Moreover, c-myc expression was dramatically reduced already after 3 h of incubation with calcitriol, making c-myc a very early response gene to calcitriol. C-myc is known to play an important role in cell proliferation. The reduced number of high affinity EGF-receptors after 48 h as found by Matsumoto et al. (15) and the overall down-modulation of mRNA synthesis following treatment for 48 h observed by us (Fig. 3) is therefore probably more an indirect consequence of growth arrest induced by selective inhibition of c-myc expression, than a specific effect on the other genes.

In conclusion, in accordance with the literature, calcitriol arrests KC in their growth. This growth arrest is followed by differentiation. Calcitriol has no specific effect on stimulus-induced mRNA expression of chemokines and the adhesion molecule ICAM-1, nor on the binding of activated T-cells to keratinocytes.

ACKNOWLEDGEMENTS

Dr R. Sager (huGRO), Dr A.B. Gottlieb (γ-IP-10) and Dr V.M. Dixit (IL-8, ICAM-1, cyclophillin and β-actin) are kindly acknowledged for providing c-DNA probes.

REFERENCES

1. Fry L. Psoriasis. Br J Dermatol 1988; 119: 445–461.
2. Valdimarsson H, Baker BS, Jonsditte I, Fry L. Psoriasis: a disease of abnormal keratinocyte proliferation induced by T-lymphocytes. Immunol Today. 1986; 7: 256–259.
3. Stoof TJ, Mitra RS, Sarma V, Dixit VM, Nickoloff BJ. Keratinocyte activation following T-lymphocyte binding. J Invest Dermatol 1992; 98: 92–95.
4. Gottlieb AB, Luster AD, Posnett DN, Carter DM. Detection of γ-interferon-induced protein (IPlO) in psoriatic scales. J Exp Med 1988; 168: 941–948.
5. Oppenheim JJ, Zachariae COC, Mukaida N, Matsushima K. Properties of the novel proinflammatory supergene "intercrine" cytokine family. Ann Rev Immunol 1991; 9: 617–648.
6. Gearing AJH, Fincham NJ, Bird CR, Wadhwa M, Maeger A., Cartwright JE, Camp RDR. Cytokine in the skin lesions of psoriasis. Cytokine 1990; 2: 68–75.
7. Schroder JM, Gregory H, Young J, Christophers E. Neutrophil activating proteins in psoriasis. J Invest Dermatol 1992; 98: 241–247.
8. Milde P. Vitamin D und die Haut, neue Aspekte und Perspektiven. Der Hautarzt 1991; 42: 671–672.
9. Larsen CG, Kristensen M. Paludan K, Deleuran B, Thomson MK, Zachariae COC, Kragballe K, Matsushima M, Thestrup-Pedersen K. 1,25 (OH)2 D3 is a potent regulator of interleukin-1 induced interleukin-8 expression and production. Biochem Biophys Res Commun 1991; 176: 1020–1026.
10. Bruynzeel I, Van der Raaÿ EMH, Boorsma DM, De Haan P, Scheper RJ, Kraal G, Willemze R. Spectrophotometric quantitation of cell-cell adherence by an enzyme-linked immuno-cell adhesion assay. J Immunol Methods 1990; 132: 51–56.
11. Smith EL, Walworth NC, Holick MF. Effect of 1, 25 dihydroxy D3 on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in serum-free conditions. J Invest Dermatol 1986; 86: 709–714.
12. Wilke MS, Hsu BM, Wille JJ, Pittelkow MR, Scott RE. Biologic mechanisms for the regulation of normal human keratinocyte proliferation and differentiation. Am J Pathol 1988; 131: 171–178.
13. Anttilla HSI, Reitamo S, Erkko P, Ceska M, Moser B, Baggiolini M. Interleukin-8 immunoreactivity in the skin of healthy subjects and patients with palmoplantar pustulosis and psoriasis. J Invest Dermatol 1992; 98: 96–101.
14. Bittner B, Bleehen SS, MacNeil S. 1,25 (OH)2 vitamin D3 increases intracellular calcium in human keratinocytes. Br J Dermatol 1991; 124: 230–235.
15. Matsumoto K, Hashimoto K, Nishida Y, Hashiro M, Yoshikawa K. Growth-inhibitory effects of 1,25 dihydroxy vitamin D3 on normal keratinocytes cultured in serum-free medium. Biochem Biophys Res Commun 1990; 166: 916–923.