Effect of calcitriol on the production of T-cell-derived cytokines in psoriasis

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Summary   Although the use of vitamin D analogues in the treatment of psoriasis has been an important new development, the mechanisms of action of these drugs are not fully understood. Psoriasis results from hyperproliferation of keratinocytes, and various studies attribute a crucial role to the locally infiltrating T lymphocytes. In an attempt to add to the understanding of the mechanisms of calcitriol therapy, we determined the effect of this drug on T cells by studying its effect on proliferation and on the production of various cytokines by T-cell clones prepared from psoriatic skin after non-specific activation with the combination of phytohaemagglutinin (PHA) and phorbol myristate acetate (PMA). The addition of increasing doses (10^{-9}–10^{-5} mol/l) of calcitriol to these T cells resulted in a dose-dependent inhibition in lymphocyte proliferation and in production of the type 1 cytokines IFN-γ and IL-2, the type 2 cytokines IL-4 and IL-5. The general cytokines TNF-α and GM-CSF were not significantly inhibited. These data suggest that calcitriol is involved in the treatment of psoriasis via inhibition of the expansion, and cytokine production, of skin-infiltrating T lymphocytes.

A major role of 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$ or calcitriol) is the regulation of calcium metabolism in humans.[1,2] In addition, calcitriol affects the skin via its effect on keratinocytes. *In vitro* studies have shown the occurrence of high-affinity low-capacity receptors for this hormone in human keratinocytes.[3] Incubation of human keratinocytes with calcitriol induced their morphological differentiation and resulted in an increased transglutaminase activity and a decreased proliferation in these cells.[4] Furthermore, calcitriol modulates immune cells as substantiated by studies indicating the local production of calcitriol by macrophages[5] and the demonstration of specific receptors for vitamin D_3 in monocytes and activated lymphocytes.[6]

Authors examining the activity of calcitriol on human peripheral blood mononuclear cells[7] have revealed that the compound is a potent inhibitor of phytohaemagglutinin (PHA)-stimulated lymphocyte proliferation. It was also found that the production of the type 1 cytokines interleukin-2 (IL-2) and interferon-γ (IFN-γ)

by lymphocytes was inhibited by calcitriol in a dose-dependent fashion.[8]

Several groups have shown that calcitriol applied either systematically or locally is useful in the treatment of psoriasis.[9,10] The exact mechanisms of action of calcitriol in psoriasis are not known, but inhibition of the production of cytokines produced by keratinocytes and lymphocytes may be crucial.[11] One potential target is the population of skin-infiltrating T cells, because these cells seem to play a part in the pathogenesis of psoriasis. T cells are found to be infiltrated in psoriatic skin and cyclosporin A, which is a selective inhibitor of T-cell proliferation and cytokine production, reduces the severity of the disease.[12]

The way that T cells contribute to psoriasis is not exactly known. T cells can be functionally categorized as cells secreting predominantly the type 1 cytokines IL-2 and IFN-γ (Th1 cells), cells predominantly producing type 2 cytokines IL-4, IL-5 and IL-10 (Th2 cells), and cells producing both type 1 and type 2 cytokines (Th0 cells), whereas all these cell types produce cytokines such as TNF-α and GM-CSF. Many of these cytokines are candidates to interact with epithelial cells. The current literature regarding the existence of a distinct cytokine pattern in lesional psoriatic skin is confusing. Some

authors report a correlation between the development of a psoriatic lesion and the accumulation of type 1 T-helper lymphocytes[13,14] but, in other studies, including our previous study, the authors did not find an association with the Th1 or Th2 pattern.[15,16]

In the present study we, therefore, examined the effect of calcitriol on T cells in more detail, by analysing the effect on T-cell proliferation and the production of a broad panel of T-cell cytokines, including the type 1 cytokines IL-2 and IFN-γ, the type 2 cytokines IL-4 and IL-5, and the more generally produced cytokines TNF-α and GM-CSF. Previously, Muller et al.[17] and Lemire et al.[18] reported on specific inhibition of proliferation and IL-2 or IFN-γ production of human T-cell lines/clones by calcitriol. We used, for this study, T-cell clones prepared from psoriatic lesions, allowing the analysis of the effect of calcitriol on the proliferation and cytokine production of psoriasis-associated T cells. The study demonstrates that calcitriol inhibits the proliferation and the production of the type 1 and type 2 cytokines of psoriatic T cells.

Materials and methods

T-cell clones, reagents and culture media

Lesional skin biopsy specimens ($n = 2$) were obtained from the forearm of six patients suffering from chronic plaque psoriasis. No local or systemic therapy had been given to the patients for 6 weeks before taking the biopsies.

The preparation of the T-cell clones used in this study has been described earlier.[19] T-cell clones were maintained in Iscove's modified Dulbecco medium (IMDM) (GIBCO, Paisley, Scotland), supplemented with 10% pooled complement-inactivated normal human serum (Central Laboratory Blood Transfusion Service, Amsterdam, The Netherlands), rIL-2 (20 U/ml) (Cetus Corp., Emeryville, California, U.S.A.) and gentamicin (80 μg/ml) and were restimulated every second week using phytohaemagglutinin (PHA), irradiated allo-peripheral blood mononuclear cells and JY cells as stimulants. When cells were stimulated for assaying cytokine production, human serum was replaced by 10% fetal calf serum (FCS) (HyClone Laboratories Inc., Logan, UT, U.S.A.) and the medium further supplemented with 35 μg/ml human transferrin (Behring-Werke, Magdeburg, Germany), 1·75 IE/ml human insulin (Actrapid, Novo Nordish A/S, Bagsvaerd, Denmark) and 3·5 μl/l β-mercaptoethanol (Merck, Munich, Germany). The Epstein–Barr virus-transformed human

B-cell line JY was maintained in IMDM containing 5% FCS. All cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Calcitriol provided by Solvay Duphar BV (Weesp, The Netherlands) was dissolved in 96% ethanol to a stock concentration of 1×10^{-3} mol/l and stored at -20°C. The various concentrations (10^{-9}–10^{-5} mol/l) were obtained by diluting the stock solution in IMDM. A vehicle control was included in all experiments. The final ethanol concentration was 0·0001%–1% in the culture medium.

The cell viability was measured by the XTT colorimetric assay (Boehringer Mannheim Biochemica, Germany, #1465015).

T-cell proliferation assay

T-cell clones (2×10^4/well) were washed three times in Hanks' balanced salt solution (HBSS; GIBCO, Paisley, Scotland) + 2% FCS and stimulated with 1 μg/ml phytohaemagglutinin (PHA, Difco, Detroit, MI, U.S.A.) and 5 ng/ml phorbol myristate acetate (PMA, Sigma Chemical Co., St Louis, MO, U.S.A.) in the presence of 10^{-5}–10^{-9} mol/l calcitriol in Costar 96-well flat-bottomed culture plates. Cells were cultured for 40 h, the last 16 h in the presence of 0·3 μCi/well of [^{3}H] thymidine (TdR, Radiochemical Centre, Amersham, U.K.). Incorporation of [^{3}H] TdR was determined by liquid scintillation spectroscopy and expressed as mean counts per minute (c.p.m.) of triplicate cultures.

Assays for cytokine production

Cytokine production assays were performed in 96-well flat-bottomed microtitre plates (Costar) 10 days after the last restimulation. 10^5 T cells were stimulated with 1 μg/ml PHA and 5 ng/ml PMA together with 10^{-5}–10^{-9} mol/l calcitriol in a volume of 200 μl per well. Controls were supernatants of cells cultured in the medium alone. Cell-free supernatants were harvested after 24 h, stored immediately at -20 °C, and thawed before testing. All experiments were performed twice in triplicate. The measurement of IL-4 and IFN-γ levels in the supernatants was performed with specific solid-phase sandwich ELISA systems, as described elsewhere.[20,21] Measurements of GM-CSF in supernatants were performed with sandwich ELISA composed of monoclonal antibody from Pharmingen Diagnostics BV, Uithoorn, The Netherlands 18581D. The levels of IL-2, IL-5 and TNF-α in the supernatants were determined by using the IL-2-dependent murine CTLL-2 cell
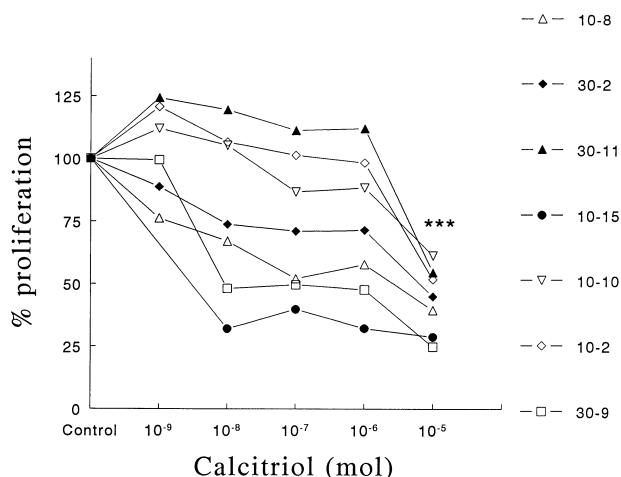
Figure 1. Proliferation by seven lesional skin T-cell clones following PHA (1 μg/ml) and PMA (5 ng/ml) stimulation for 40 h in the presence or absence of calcitriol. Each symbol and the consecutive numbers represent a distinct T-cell clone. Results are expressed as per cent of control values. Calcitriol dose-dependently inhibits proliferation (ANOVA, $P = 0.0004$). ***$P < 0.001$, multiple comparisons test, control vs. dose calcitriol.

line,[22] the IL-5-dependent cell line B13[23] and the cell killing of the L929 fibroblast cell line in the presence of TNF-α,[24] respectively. The tested agents, at the concentrations used, did not interfere with the proliferation of the CTLL-2, B13 or L929 cells.

Statistical analysis

Results of measurements were statistically analysed using one-way and repeated measures analysis of variance (ANOVA) and the Student–Newman–Keuls multiple comparisons test.

Results

To determine the effect of calcitriol on proliferation and cytokine production of T-cell clones, representative clones were stimulated with the combination of PHA and PMA in the presence of 10^{-9}–10^{-5} mol/l calcitriol. In some experiments similar concentrations of hydrocortisone and cyclosporin A served as a control and both compounds caused dose-dependent inhibition of all tested cytokines (data not shown).

Calcitriol at the concentration of 10^{-8}–10^{-5} mol/l dose-dependently inhibited the proliferation of the tested T-cell clones (Fig. 1) (ANOVA, $P = 0.0004$). The decrease in proliferation was not due to a decrease in cell survival, as the cell viability as measured by the XTT

colorimetric assay was similar in all test conditions (data not shown).

As reported before, the cytokine profile of stimulated psoriatic T-cell clones varied considerably, with some clones that did not produce either type 1 or type 2 cytokines.[16] However, in all cases when these cytokines were produced, calcitriol dose-dependently inhibited the production of the type 1 cytokines IL-2 (ANOVA, $P < 0.0001$) and IFN-γ (ANOVA, $P = 0.0031$) as well as the type 2 cytokines IL-4 (ANOVA, $P = 0.0268$) and IL-5 (ANOVA, $P < 0.0001$) (Fig. 2). Calcitriol did not significantly inhibit the production of the non-Th1/Th2-associated cytokines TNF-α (ANOVA, $P = 0.7498$) and GM-CSF (ANOVA, $P = 0.0692$) (Fig. 3).

Discussion

Since the discovery of receptors for calcitriol in human monocytes and activated, but not resting T lymphocytes,[6,25] much attention has been focused on the participation of the vitamin D$_3$–endocrine system in immunoregulatory processes. In our experiments, calcitriol was capable of suppressing lymphocyte proliferation. The maximum inhibition of lectin-induced lymphocyte proliferation was 70%. From the work of Rigby *et al.*[7] we know that specific receptors for vitamin D are not detectable in T lymphocytes until 24 h after lectin stimulation. The absence of an inhibitory effect on proliferation of some of the T-cell clones, therefore, may be explained by the lack of receptors for calcitriol in these cells at the onset of the experiment.

Our previous study showed that the skin-infiltrating lymphocytes do not show shifts towards either a Th1 or a Th2 cytokine production profile.[16] Therefore we examined the effect of calcitriol on proliferation and the production of cytokines by Th0, Th1 and Th2 cells. Cytokine production was inhibited by calcitriol in a dose-dependent fashion with an effect at concentrations as low as 10^{-9} mol/l calcitriol in the culture medium. The physiological concentration of calcitriol in normal human serum is 10^{-10} mol/l.[26] Calcitriol was not toxic for T cells in any concentration used, since XTT assay showed similar cell viability in all test conditions.

Several studies prompted that the effect of calcitriol on cytokine synthesis is receptor-mediated. The receptor is a specific nuclear receptor (vitamin D receptor), which is related to the steroid, thyroid and retinoic acid receptor superfamily. Vitamin D receptors are expressed not only by lymphocytes but by keratinocytes, monocytes and macrophages as well. The receptor complex interacts with DNA sequences regulating
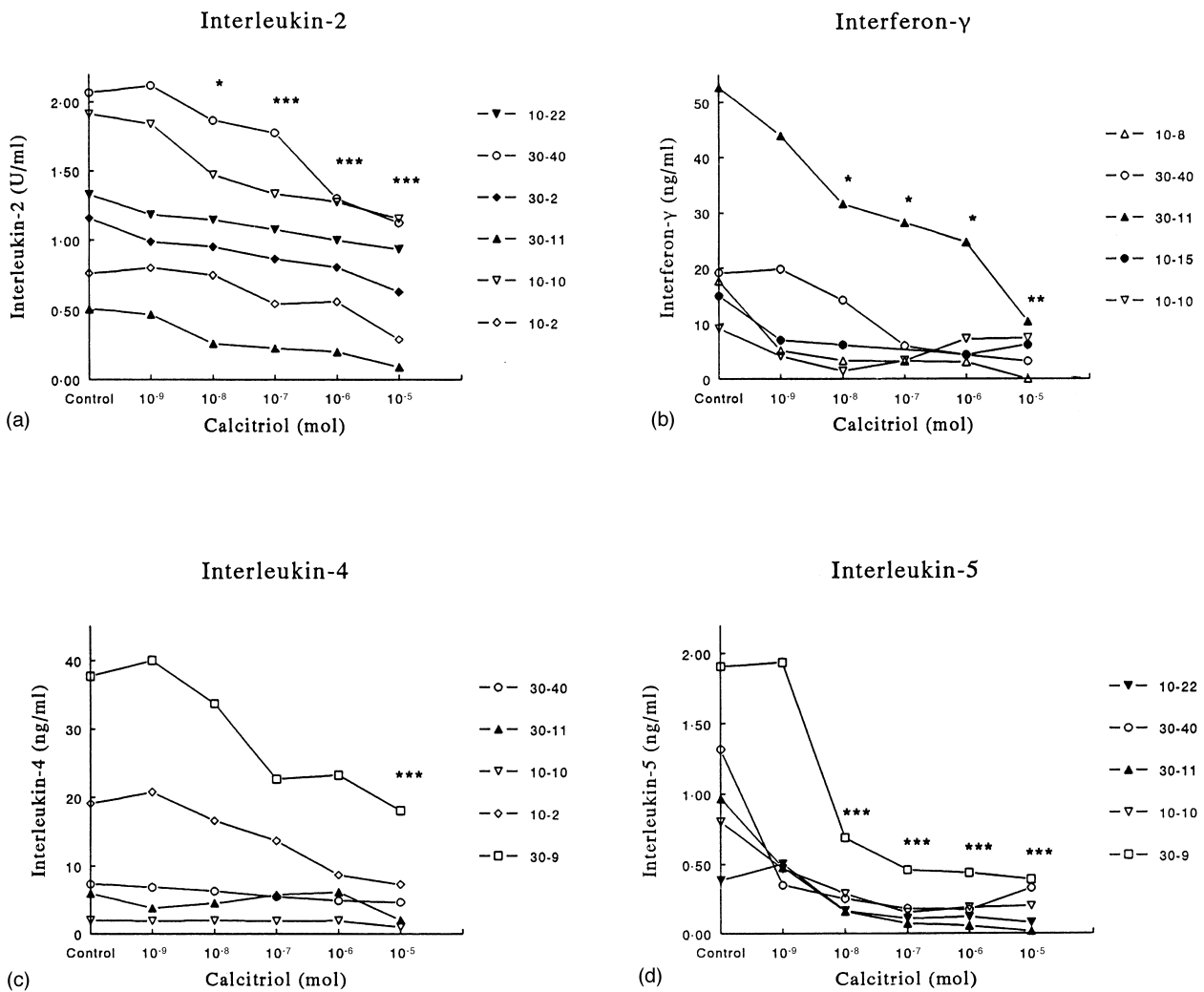
Interleukin-2

Interferon-γ

Interleukin-4

Interleukin-5

Figure 2. (a–d) Effect of calcitriol on T-cell derived cytokines following PHA (1 μg/ml) and PMA (5 ng/ml) stimulation for 24 h in the presence or absence of calcitriol. Calcitriol dose-dependently inhibits cytokine production (ANOVA IL-2, $P < 0\cdot0001$; IFN-γ, $P = 0\cdot0031$; IL-4, $P = 0\cdot0268$; IL-5, $P < 0\cdot0001$). *$P < 0\cdot05$; **$P < 0\cdot01$; ***$P < 0\cdot001$.

synthesis of mRNA involved in cell growth and differentiation.[27] More than 400 vitamin D_3 responsive genes have been reported recently.[28]

The study of Tsoukas *et al.*[29] suggested that calcitriol-mediated inhibition of lymphocyte proliferation and IL-2 synthesis was dependent on the presence of monocytes in cell cultures. Our experiments are inconsistent with such a requirement of accessory cells (B cells, monocytes) for calcitriol-mediated inhibition because they were performed with pure T-cell clones stimulated in the absence of accessory cells. Similar inhibition of cytokine production and proliferation was observed in peripheral blood lymphocytes and keratinocytes.[3,30] Lemire *et al.* and Muller *et al.*

found some selectivity in cytokine inhibition; in their systems calcitriol preferentially inhibited Th1-type cytokines.[17,18] The authors of these studies used different cloning procedures and lower calcitriol concentrations. The spectrum of cytokines they investigated was generally smaller and the studies had excluded IL-5 or the general cytokines TNF-α and GM-CSF. We cloned T cells directly from psoriatic lesions and by lectin stimulation we were able to detect type 1 and type 2 cytokines as well. In accordance with Baadsgaard,[27] we could demonstrate direct inhibition of type 1 and type 2 cytokines by calcitriol.

The present study indicates that calcitriol controls immunological reactions by a generalized down-regulation
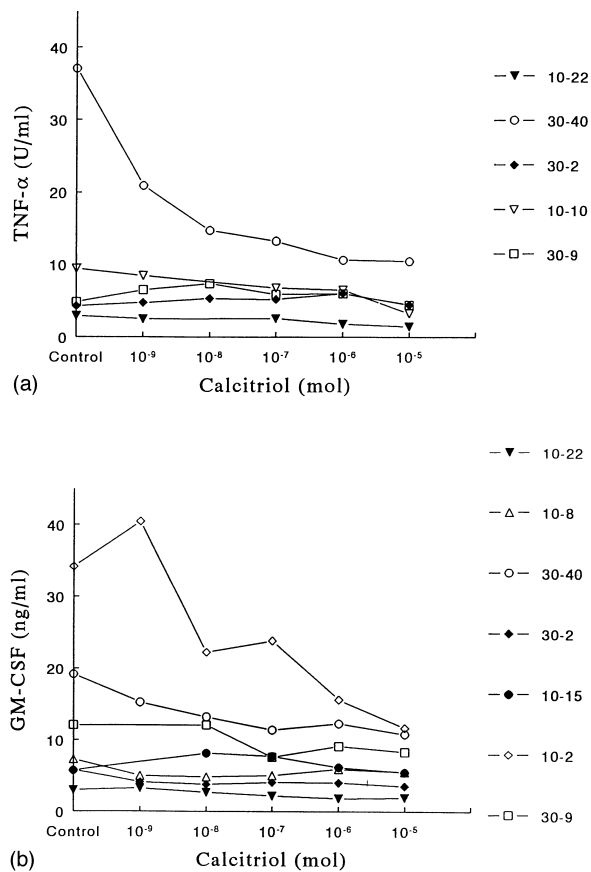
Figure 3. Effect of calcitriol on (a) TNF-α and (b) GM-CSF production following PHA (1 μg/ml) and PMA (5 ng/ml) stimulation; T cells were incubated for 24 h in the presence or absence of calcitriol. The inhibition of cytokines was not significant (ANOVA TNF-α, $P = 0.7498$; GM-CSF $P = 0.0692$).

of T-cell activation. Calcitriol is a potent drug for the treatment of psoriasis. Thus, a possible mechanism of action of calcitriol as a drug is its action on T cells, i.e. the downregulation of local expansion and the production of one or more cytokines important in the pathophysiology of psoriasis.

Acknowledgments

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