Effects of 1,25 Dihydroxyvitamin D_3 and Calcium on Growth and Differentiation and on *c-fos* and p53 Gene Expression in Normal Human Keratinocytes

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Calcium enhances keratinocyte differentiation, and 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is both antiproliferative and prodifferentiative in many cell types, including normal human keratinocytes. In the present study, we examined the combined effects of calcium and $1,25(OH)_2D_3$ on parameters of growth and differentiation and on *c-fos* and p53 gene expression in normal human keratinocytes. Exposure of normal human keratinocytes to $1,25(OH)_2D_3$ markedly reduced [^{3}H] thymidine incorporation and cell number at low and high medium Ca^{++} concentrations. Simultaneously, cells in the G_0/G_1 phase of the cell cycle increased significantly and those in the S phase fell precipitously. $1,25(OH)_2D_3$ and calcium also induced keratinocyte differentiation independently, as assessed by immunocytochemistry and by induction of involucrin mRNA. Both Ca^{++} and $1,25(OH)_2D_3$ were shown, by nuclear run-on assays, to increase involucrin gene transcription. A rapid, transient elevation in *c-fos* protooncogene expression preceded these effects when epidermal growth factor was present alone. When $1,25(OH)_2D_3$ was added to quiescent keratinocytes, there was a marked augmentation of *c-fos* mRNA accumulation at low and high medium Ca^{++} concentrations. Varying medium Ca^{++} concentrations had no effect on *c-fos* mRNA levels. Increasing medium Ca^{++} concentrations from 0.15 to 2.0mM produced marked elevations of p53 mRNA accumulation and of the rate of p53 gene transcription, whereas $1,25(OH)_2D_3$ had no effect.

These results, therefore, suggest that $1,25(OH)_2D_3$ and calcium act in concert to modulate the expression of two important cell-cycle–associated genes, which may be important components in the initial programming of growth and differentiation of normal human keratinocytes. *J Invest Dermatol 103:323–329, 1994*

1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is believed to exert its action through an interaction with its hormone-receptor that complexes with DNA and concentrates the hormone in the nucleus [1] similar to other steroid hormones [2]. Several target genes, such as the proto-oncogenes *c-myc* and *c-fos* probably play an important role in the regulation of growth and differentiation of several cell types [3]. Activation of *c-myc* is associated with cell proliferation [4], whereas activation of *c-fos* is thought to occur in association with cellular differentiation [5,6]. Elevation of medium calcium concentrations to 1.0mM or more inhibits growth strongly and promotes differentiation of keratinocytes in culture [7]; $1,25(OH)_2D_3$ potentiates these effects [8,9]. Recent evidence shows that the inhibition of the growth of normal human keratinocytes by $1,25(OH)_2D_3$ is preceded by marked inhibition of *c-myc* mRNA [10]. In addition to proto-oncogenes, tumor suppressor genes such as p53 can also alter cell growth [11]. Growth inhibition is observed when p53 is stably transfected into cancer cell lines. Mutations along the p53 gene, which result in its decreased suppressor activity, are found in a wide variety of cancers [12]. p53 is also expressed in normal cells and is thought to play a role in normal cell growth [13–15].

At present we do not know whether the effect of calcium and $1,25(OH)_2D_3$ on keratinocyte growth and differentiation is accompanied by changes in the expression of the cell-cycle–associated genes, *c-fos* and p53.

We therefore analyzed the effect of calcium and $1,25(OH)_2D_3$ alone and in combination on parameters of cell growth and differentiation and on expression of *c-fos* and p53 genes.

MATERIALS AND METHODS

Culture of Normal Human Keratinocytes We isolated normal human keratinocytes from skin tissue removed during breast reduction, according to the method of Boyce and Ham [16]. In brief, epidermal tissue was separated from dermal tissue following a 3-h collagenase digestion. Single cells were released from the epidermis using trypsin and were suspended in keratinocyte growth medium (KGM), the complete medium for clonal growth of keratinocytes. KGM consists of keratinocyte basal medium (KBM Clonetics Corp., San Diego, CA) containing 0.15 mM Ca^{++} supplemented with the following growth factors (GFs): 10 ng/ml epidermal growth factor (EGF, Sigma), 5 μg/ml insulin (Sigma, St. Louis, MO), 0.5 μg/ml hydrocortisone (Clonetics), 0.4% (W/V), and bovine pituitary extract (BPE, Clonetics). It provided maximal proliferation without differentiation. Cells grown in these conditions were then seeded at a density of 2.5×10^4 cells/well in 6-well cluster plates in KGM for 24 h. Following a 24 h incubation in KBM and 0.15 mM Ca^{++} (basal conditions), the medium was replaced at time 0 with KGM containing EGF (10 ng/ml) in which Ca^{++} concentrations varied between 0.15 and 2.0 mM, verified by direct measurement by atomic absorption spectrophotometry. Incubations were then continued for up to 96 h with or without 10^{-8} M $1,25(OH)_2D_3$.

Manuscript received September 16, 1993; accepted for publication March 30, 1994.

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Abbreviations: BPE, bovine pituitary extracts; GF, growth factors; KBM, keratinocyte basal medium; KGM, keratinocyte growth medium; MEM, minimal essential medium.

0022-202X/94/$07.00

Cell Counts We dispersed cells after trypsinization and counted an aliquot in a Coulter counter (Coulter Electronics, Beds, U.K.) at timed intervals up to 96 h after medium change. Remaining cells were centrifuged at low speed (600 × g), rinsed with phosphate-buffered saline, and lysed with a mixture of 4 M guanidium thiocyanate, 25 mM trisodium citrate, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 M β-mercaptoethanol (GTC mixture). GTC extracts were stored at −70°C for later RNA analysis by Northern blot hybridization.

[^{3}H]Thymidine Incorporation Into DNA We replaced the medium at 22 h and at 94 h with calcium free minimal essential medium (MEM) (Gibco, Grand Island, NY) supplemented with either 0.15, 0.5, or 2.0 mM Ca^{++} concentration and 1 μCi/ml of [^{3}H]thymidine (New England Nuclear, Boston, MA). Following a 2 h incubation at 37°C, the medium was aspirated, and cells were washed twice with cold Hanks' balanced salt solution and incubated 15 min with 1 ml of cold 10% trichloroacetic acid (TCA) to precipitate protein. After aspirating the TCA, protein precipitable material was dissolved in 1 ml of 1 N NaOH and the ^{3}H content in an aliquot was determined by liquid scintillation spectrometry in an LKB β radiation counter. Cell numbers were counted and the [^{3}H]thymidine counts/min (cpm) were corrected for cell number, with final values expressed as cpm/10^4 cells.

Immunocytochemistry The cellular content of specific keratins was determined by immunocytochemistry, using a polyclonal antiserum to keratins, predominantly of MW 56.5 kD and 65–67 kD (Dimension Labs, Mississauga, Ontario, Canada). These keratins have been immunolocalized to the suprabasal layers of human epidermis and have been characterized as markers for skin-type differentiation [17,18]. Cells seeded in four chamber glass slides (Gibco) in KGM were grown to approximately 30% confluency. The medium was then changed to keratinocyte basal medium (KBM) (basal conditions). After 24 h in basal conditions, the medium was replaced with KBM containing 10 ng/ml EGF and either 0.15 or 2.0 mM Ca^{++}, with or without 10^{-8} M $1,25(OH)_2D_3$. Incubation continued for 5 d with a medium change after 3 d. Cells were then fixed in 95% ethanol for 5 min, rinsed with distilled water, and stained by a modification of the three-layer peroxidase-antiperoxidase technique [19].

Flow Cytometry Cells were grown in KGM until they reached 50% confluency. Following a 24 h incubation in KBM, the medium was replaced with KBM containing EGF (10 ng/ml) at low (0.15 mM) or high (2.0 mM) Ca^{++} concentration and were incubated in the presence or absence of 10^{-8} M $1,25(OH)_2D_3$. Twenty-four hours later they were analyzed by flow cytometry. Following trypsinization cells were centrifuged at low speed (600 × g), rinsed once with phosphate-buffered saline (PBS), and stained according to the technique of Vindeløv [20]. Briefly, the pellet was resuspended in 1 ml of 3.5 mM Tris, 7.5 μM propidium iodide (Calbiochem), 0.1% nodinet P40 (Sigma), 700 μl RNAse (Boehringer, Mannheim, Canada), and 10 mM NaCl. The solution was added dropwise while vortexing. After standing at least 10 min on ice, the nuclei were analyzed in a FACScan (Becton Dickinson Inc., Oxnard, CA), which sorts and plots the number of cells against the relative fluorescence intensity. The calculation of the percentage distribution in various phases of the cell cycle was performed with Cell Fit software (Becton Dickinson Inc.), using a sum of broadened rectangles fit.

RNA Analysis For Northern Blot analysis, we purified GTC extracts by cesium chloride gradient centrifugation [21] and electrophoresed 10 μg of total RNA on a 1.1% agarose-formaldehyde gel. RNA was transferred by capillary blotting onto a nylon membrane (Nytran). The filters were air-dried, baked at 80°C for 2 h, and then hybridized with probes labeled with ^{32}P dCTP (IC Biomedical Canada LTD) by the random primer method (Amersham Canada LTD, Ontario, Canada). After incubation at 42°C for 24 h, filters were washed successively in 1 × SSC, 1% SDS for 15 min at room temperature, and 0.1 × SSC, 0.1% sodium dodecylsulfate twice for 30 min at 55°C (1 × SSC is 0.15 M sodium chloride, 0.015 M trisodium citrate). Autoradiography of filters was carried out at −70°C using Kodak XAR films (Eastman Kodak Co., Rochester, NY) and two intensifying screens. The intensities of the observed bands were analyzed by laser densitometry (Ultroscan XL, LKB).

DNA Probes a) Involucrin was used as a differentiation marker of keratinocytes. Involucrin is produced abundantly during terminal differentiation of keratinocytes and works as a precursor of a cross-linked envelope during terminal differentiation [22–25]. A 2.1-kb fragment of the human involucrin gene, containing the entire coding region, was used as a probe [26].

b) *c-fos*: the EcoR1-SalI restriction fragment encoding *c-fos* released from the plasmid *p-fox* BS [27] was used as a probe.

c) p53: a 2.0 KB Bam H1 restriction fragment of p53 cDNA was used [28].

d) Cyclophilin: filters were also probed with a 800 bp BamH1, restriction fragment of rat cyclophilin [29] as a control for the amount of RNA loaded.

e) GAPDH: a rat Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was prepared from a PstI digested pRGAPDH13 plasmid [30].

***In vitro* Nuclear Run-on Assays** Relative transcription rates of involucrin, p53, and cyclophilin genes were measured using a nuclear run-on assay [31,32]. Nuclei were prepared from 10–20 × 10^6 cells prepared as described in paragraph 1 in the presence or absence of EGF, $1,25(OH)_2D_3$ and in low (0.15 mM) or high (2.0 mM) Ca^{++} concentrations. Cells were scraped into ice-cold PBS, pH 7.4, pelleted at 4°C, and lysed with Nodinet P40 lysis buffer [0.3M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM HEPES (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 14 mM β-mercapto ethanol and 0.2% Nodinet P40]. After 8 min on ice, nuclei were pelleted at 800 × g. They were rinsed once with 1 ml nuclei storage buffer [50% glycerol, 20 mM Tris (pH 7.9), 75 mM NaCl, 0.5 mM EDTA, 0.85 mM dithiothreitol (DTT), and 0.125 mM phenylmethylsulfoxide (PMSF)], snap frozen in liquid nitrogen, and stored at −80°C until assay. Run-on reactions were carried out at 30°C in 300 mM $NH_4(SO_4)_2$, 100 mM Tris HCl (pH 7.9), 4 mM $MgCl_2$, 4 mM $MnCl_2$, 50 mM NaCl, 0.4 mM EDTA, 1.2 μM DTT, 0.1 mM PMSF, 10 mM creatine phosphate, 29% glycerol, 150 μCi [^{32}P] UTP, 650 Ci/mmol (ICN, Mississauga, Ontario, Canada), and 1.5 mM each of CTP, ATP, and GTP (Boehringer) for 45 min. Reactions were quenched with tRNA and treated with (RNAse-free) DNAse and proteinase-K and phenol-chloroform-isoamyl alcohol extracted. ^{32}P labeled transcripts were spun, column chromotographed through sephadex G50, TCA precipitated, NaOH treated, and ethanol precipitated. DNA inserts (0.15 μg) of involucrin, p53, and GAPDH prepared as described above were NaOH denatured, slot blotted (Hybrislot, Gibco/BRL, Burlington, Ontario, Canada) and hybridized with 2 × 10^7 cpm ^{32}P labeled transcripts in 50% formamide, 50 mM HEPES (pH 7.3), 0.75 M NaCl, 2 mM EDTA, 0.5% SDS, 10 × Denhardt's, and 200 μg/ml salmon sperm DNA for a minimum of 40 h. In any single experiment, equal number of counts were used for all conditions. Nytran filters were exposed to autoradiographic film and quantitation of the bands was done by laser densitometry.

Statistical Analysis Results are expressed as the mean ± SEM of replicate (at least triplicate) determinations and statistical comparisons are based on analysis of variance or the Student t test. A probability value of 0.05 was considered significant.

RESULTS

Effect of Ca^{++} and $1,25(OH)_2D_3$ On Cell Proliferation and [^{3}H]Thymidine Incorporation Normal human keratinocytes were incubated in the presence and in the absence of 10^{-8} M $1,25(OH)_2D_3$ in 0.15, 0.5 or 2.0 mM Ca^{++}. In the absence of $1,25(OH)_2D_3$ cells responded to 2.0 mM Ca^{++} by decreasing their growth rate **(Fig 1*C*)**; no difference was noted between 0.15 and 0.5 mM Ca^{++} **(Fig 1*A,B*)**. Cells treated with $1,25(OH)_2D_3$ showed a markedly reduced growth rate compared to untreated cells at all three calcium concentrations tested. Similarly [^{3}H]thymidine incorporation was inhibited by 10^{-8} M $1,25(OH)_2D_3$ at each of the three Ca^{++} levels, whereas ambient Ca^{++} had to be raised to 2.0 mM to see inhibition in the absence of $1,25(OH)_2D_3$ **(Fig 1*D,E,F*)**. Similar inhibitory effects of $1,25(OH)_2D_3$ on growth rate and [^{3}H]thymidine incorporation of rapidly proliferating keratinocytes were noted at low (0.15 mM) and intermediate (0.5 mM) Ca^{++} concentrations. However, at a high Ca^{++} concentration (2.0 mM), $1,25(OH)_2D_3$ totally abolished cell proliferation.

Effect of $1,25(OH)_2D_3$ and Calcium On Keratinocyte Differentiation

Immunocytochemistry: Immunocytochemical studies using the 56.5 kD/65–67 kD keratin pair as markers of keratinization showed that $1,25(OH)_2D_3$ induced staining at low Ca^{++} concentration (0.15 mM) **(Fig 2*B*)**; both the intensity of staining and the number of stained cells were maximally increased at high (2.0 mM) Ca^{++} with $1,25(OH)_2D_3$ **(Fig 2*C*)**.

Keratinocyte Differentiation Assessed By Involucrin mRNA Expression Involucrin was seen as a faint message in nondifferentiated keratinocytes, incubated in KBM, as a single 2.1-kB transcript. Addition of $1,25(OH)_2D_3$ in the presence of low calcium (0.15 mM) caused a marked increase in involucrin mRNA, maximal at 48 h **(Fig 3*A*)**. Simultaneous addition of 2.0 mM calcium and $1,25(OH)_2D_3$ produced a marked increase of involucrin mRNA at 24 h that remained stable thereafter **(Fig 3*B*)**. However, the maximal level of expres-

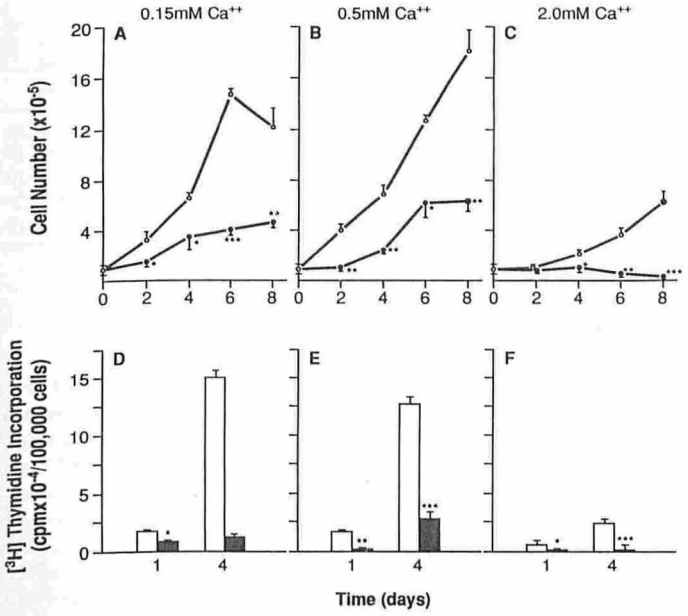

Figure 1. Effect of Ca^{++} and 1,25 dihydroxyvitamin D_3 on cell number and [^{3}H]thymidine uptake in quiescent keratinocytes stimulated with EGF supplemented KBM. EGF alone (○, □) or with 10^{-8} M $1,25(OH)_2D_3$ (●, ■). EGF in 0.15 mM Ca^{++} (*A,D*), in 0.5 mM Ca^{++} (*B,E*), or in 2.0 mM Ca^{++} (*C,F*). Cells were counted at the times indicated and [^{3}H]-thymidine uptake was measured as described in *Materials and Methods*. Results are expressed as the mean ± SEM of six determinations. *Asterisks*, significant differences from corresponding incubations performed in the absence of $1,25(OH)_2D_3$. *$p < 0.01$ **$p < 0.005$ ***$p < 0.001$.

sion achieved at 48 h was not significantly different between 2.0 mM calcium and $1,25(OH)_2D_3$ added separately or with the combination of $1,25(OH)_2D_3$ and 2.0 mM calcium. Finally, there was a marked increase in involucrin mRNA with addition of 2.0 mM calcium to the culture medium; it was maximal at 48 h **(Fig 3*C*)**.

Effect of $1,25(OH)_2D_3$ On The Cell Cycle Using flow cytometry, we next examined the effect of $1,25(OH)_2D_3$ on the progression of normal human keratinocytes through the cell cycle. In the quiescent state the majority of cells were in G_0/G_1 phase. The addition of EGF for 24 h resulted in a shift into S phase. The addition of 10^{-8} M $1,25(OH)_2D_3$ to culture medium containing 0.15 mM Ca^{++} and EGF inhibited the shift. Addition of 2.0 mM Ca^{++} along with EGF to the culture medium also inhibited the shift into the S phase. Finally, the combination of 2.0 mM Ca^{++}, 10^{-8} M $1,25(OH)_2D_3$, and EGF had a more pronounced effect than 2.0 mM calcium or $1.25(OH)_2D_3$ alone **(Table I)**.

Effect of EGF, Calcium And of $1,25(OH)_2D_3$ on *c-fos* and p53 mRNA After 24 h incubation in KBM, the medium was removed and replaced with KBM containing EGF (10 ng/ml) and Ca^{++} (0.15 or 2.0 mM) with or without 10^{-8} M of $1,25(OH)_2D_3$. EGF addition in the presence of 0.15 mM Ca^{++} produced a rapid and transient increase of *c-fos* mRNA **(Fig 4*A*)**. Addition of $1,25(OH)_2D_3$ markedly enhanced *c-fos* mRNA levels above the one seen with EGF alone as early as 30 min and up to 24 h both in low (0.15 mM) **(Fig 4*A*)** or high (2.0 mM) medium Ca^{++} concentrations **(Fig 4*B*)**. Addition of 2.0 mM calcium did not significantly alter the level nor the time course of *c-fos* mRNA expression **(Fig 4*C*)**. We next examined the effect of EGF, calcium, and $1,25(OH)_2D_3$ on p53 mRNA expression. Addition of EGF in the presence of 0.15 mM Ca^{++} produced an early (60 min) and progressive increase of p53 mRNA, maximal at 48 h **(Fig 5*A*)**. However, $1,25(OH)_2D_3$ did not alter p53 mRNA levels significantly at any of the time points analyzed whether the cells were incubated with 0.15 mM **(Fig 5*A*)** or 2.0 mM medium calcium concentrations **(Fig 5*B*)**. In contrast to the

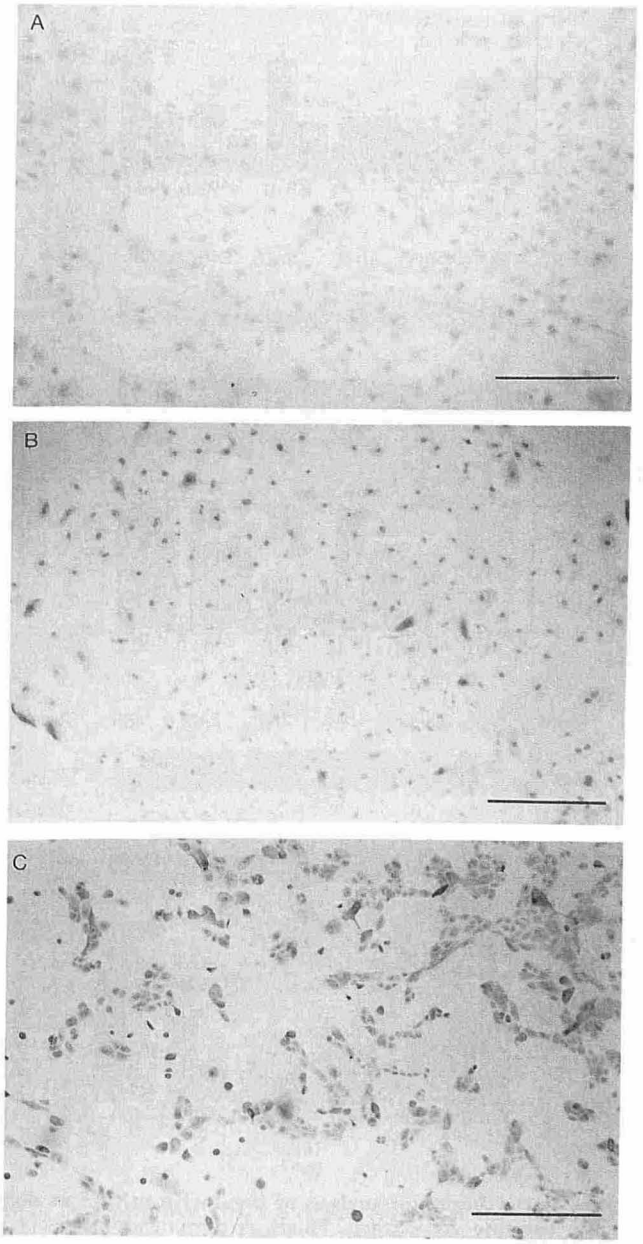

Figure 2. Effect of Ca^{++} and 1,25 dihydroxyvitamin D_3 on keratinocyte differentiation using immunocytochemical stain for the 56.5kD/65–67kD keratin pair. Keratinocytes were fixed, permeabilized, and processed for immunocytochemistry as described in *Materials and Methods*. Cells were cultured in *(A)* 0.15 mM Ca^{++}; *(B)* 0.15 mM Ca^{++} and $1,25(OH)_2D_3$; *(C)* 2.0mM Ca^{++} and $1,25(OH)_2D_3$. *Bars*, 75 μm.

absence of an effect seen on *c-fos* mRNA after addition of 2.0 mM calcium, p53 mRNA was significantly increased at 24 h and was maintained at 48 h after calcium addition **(Fig. 5*C*)**.

Effect of Ca^{++} and $1,25(OH)_2D_3$ On Involucrin And p53 Gene Transcription To determine whether the effects of these various factors were acting at the transcriptional level, we performed nuclear transcription run-on assays. After exposing the cells to 0.15 and 2.0 mM Ca^{++} with and without 10^{-8} M $1,25(OH)_2D_3$ for 2 h and 24 h, nuclear extracts were labeled and hybridized to specific target DNA sequences, as shown in **Fig 6**. Hybridization to pBR322 was absent and transcription of GAPDH, used as internal control, was unaffected by Ca^{++} or $1,25(OH)_2D_3$. By contrast $1,25(OH)_2D_3$ increased involucrin gene transcription approximately 3.5-fold at 2 h **(Fig 6*A*)** and six-fold at 24 h **(Fig 6*B*)**, but

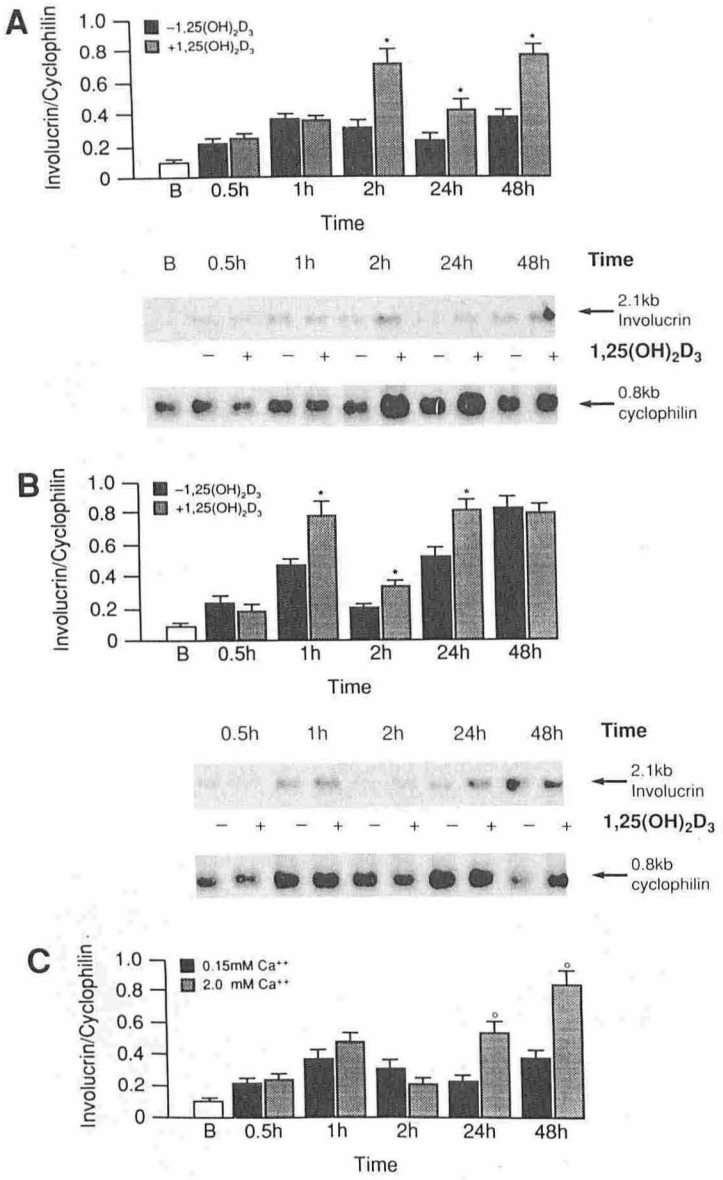

Figure 3. Northern blot analysis of involucrin mRNA in normal human keratinocyte extracts. Filters were hybridized as described in *Materials and Methods* with a ^{32}P-labeled involucrin probe and with a ^{32}P-labeled cyclophilin probe as a control for RNA loading. The upper panels represent the densitometric ratios of involucrin/cyclophilin mRNA seen in lower panels. *Arrows,* the 2.1 kb involucrin message and the 0.8 kb cyclophilin message. Influence of $1,25(OH)_2D_3$ in 0.15 mM Ca^{++} *(A)* or 2.0 mM Ca^{++} *(B)*, and the influence of Ca^{++} *(C)* on the time course of involucrin mRNA. Each lane contained 10 μg of total cellular RNA. The ratios are expressed as mean ± SEM of three different experiments. *Asterisks* represent significant differences from corresponding incubations in absence of $1,25(OH)_2D_3$, and *circles* in the presence of 2.0 mM calcium.

did not affect p53 gene transcription. Ca^{++} increased both involucrin and p53 gene transcription approximately six-fold and eight-fold respectively, at 24 h **(Fig 6*B*)**.

DISCUSSION

In this study we examined the effect of $1,25(OH)_2D_3$ and calcium on parameters of cell growth and differentiation and on *c-fos* and p53 expression in normal human keratinocytes. Previous studies showed both the existence of a specific receptor for $1,25(OH)_2D_3$ [33] and local production of the hormone by keratinocytes [34]. This steroid could therefore regulate growth and differentiation [8,9] of keratinocytes in an autocrine manner.

Table I. Effect of Calcium and $1,25(OH)_2D_3$ on EGF-Stimulated Keratinocyte Proliferation

	G_0G_1 (%)[a]	S (%)[a]
Untreated cells	77.5 ± 0.8[c]	9.2 ± 0.6[c]
EGF + 0.15 mM Ca^{++}	69.5 ± 2.0[b]	13.8 ± 0.8[b]
EGF + 0.15 mM Ca^{++} + $1,25(OH)_2D_3$	74.1 ± 1.2[b,c]	11.6 + 0.6[b,c]
EGF + 2.0 mM Ca^{++}	73.3 ± 1.1[b,c]	11.1 ± 0.7[b,c]
EGF + 2.0 mM Ca^{++} + $1,25(OH)_2D_3$	77.0 ± 2.0[c]	9.3 ± 0.6[c]

[a] Results are expressed as % ± SEM of at least six determinations.
[b] Significant difference from untreated cells, $p < 0.05$.
[c] Significant difference from EGF treated cells in 0.15 mM Ca^{++}.

We observed that $1,25(OH)_2D_3$ is a potent growth inhibitor at low (0.15 mM) and intermediate Ca^{++} concentration (0.5 mM). However, the growth inhibition observed was similar for both Ca^{++} concentrations. In contrast, the combination of 2.0 mM Ca^{++} and $1,25(OH)_2D_3$ completely abolished cell division. Therefore, it appeared that $1,25(OH)_2D_3$ initiated specific cellular events under conditions where normal human keratinocytes would normally proliferate and not differentiate. This calcium independent effect was supported by the synergistic effect of calcium and $1,25(OH)_2D_3$ seen at higher Ca^{++} concentrations. This effect is consistent with previous findings reported in normal human keratinocytes showing dose-dependent inhibition of cell growth by $1,25(OH)_2D_3$ with a minimal effective dosage of around 10^{-8} M [35], a concentration used throughout the present study. The human keratinocytes used in our study displayed cell specific arrest in the G_0/G_1 phase of the cell cycle in response to $1,25(OH)_2D_3$. The arrest in the resting phase of the cycle was early and independent of the Ca^{++} concentration, demonstrating the specificity of the effect of $1,25(OH)_2D_3$ on growth inhibition by blocking entry into the S phase of the cell cycle.

We then assessed the effect of $1,25(OH)_2D_3$ and calcium on keratinocyte differentiation. Normal human keratinocytes in culture proliferate in low Ca^{++} concentration and differentiate when ambient Ca^{++} is raised to or above 1.0 mM [7]. Epidermal differentiation has previously been extensively studied using several key markers, including keratins [36], involucrin [22,23], and fillagrin [37]. Involucrin is a 68-kd precursor protein of the keratinocyte cornified envelope [22,23] and correlates positively with the process of differentiation. Involucrin mRNA expression was recently shown to be an excellent marker of keratinocyte differentiation under the influence of calcium or phorbol esters [38]. In the present study, we analyzed involucrin expression over time in the presence of various concentrations of Ca^{++} and $1,25(OH)_2D_3$. Calcium or $1,25(OH)_2D_3$ strongly stimulated involucrin expression to similar levels, corroborating our immunocytochemical data which demonstrated positive staining of differentiated cells with markers of keratinization. These observations are consistent with the increased involucrin protein levels observed previously in differentiated cells [39,40]. Mitogenic stimuli and $1,25(OH)_2D_3$, or its metabolites, are also known to upregulate the abundance of vitamin D receptors [41,42]. This may contribute to the sustained overexpression of involucrin mRNA, in the presence of $1,25(OH)_2D_3$ and EGF, in our model. Furthermore nuclear run-on analysis demonstrated that the effect of Ca^{++} and $1,25(OH)_2D_3$ occur at the level of gene transcription. Nevertheless, it should be noted that the observed effect of $1,25(OH)_2D_3$ in the nuclear run-on assays is much more pronounced than the effect observed in the Northern blot analysis. Because steady-state mRNA levels seen on Northern blots reflect a balance between the rates of transcription and degradation, these results may suggest a relatively more rapid rate of PTHRP mRNA degradation. Further studies on involucrin mRNA stability should help clarify this issue. The transcriptional regulation of involucrin by $1,25(OH)_2D_3$ suggests the presence of Vitamin D response element(s) (VDRE(s)) in the promoter region of this gene. Such cis-acting elements have been previously identified in the osteocalcin [43] and osteopontin genes [44] but have not yet been defined in the 5′ flanking region of the involucrin gene.

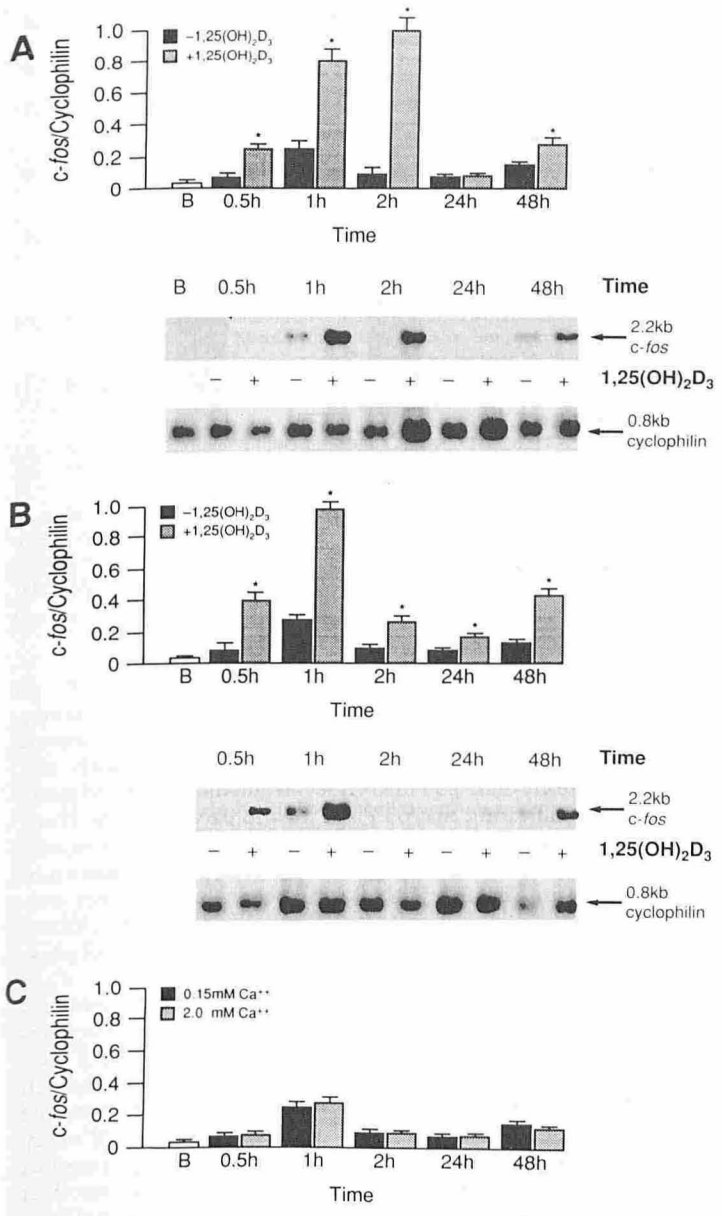

Figure 4. Northern blot analysis of *c-fos* mRNA in normal human keratinocyte extracts. Filters were hybridized as described in *Materials and Methods* with a ^{32}P-labeled *c-fos* probe and a ^{32}P-labeled cyclophilin probe as a control for RNA loading. The upper panels represent the densitometric ratios of *c-fos*/cyclophilin mRNA seen in lower panels. *Arrows*, the 2.2 kb *c-fos* message and the 0.8 kb cyclophilin message. Influence of $1,25(OH)_2D_3$ in 0.15 mM Ca^{++} *(A)* or 2.0 mM Ca^{++} *(B)*, and the influence of Ca^{++} *(C)* on the time course of *c-fos* mRNA. Each lane contained 10 μg of total cellular RNA. The ratios are expressed as mean ± SEM of three different experiments. *Asterisks* represent significant differences from corresponding incubations in absence of $1,25(OH)_2D_3$ and *circles* in the presence of 2.0 mM calcium.

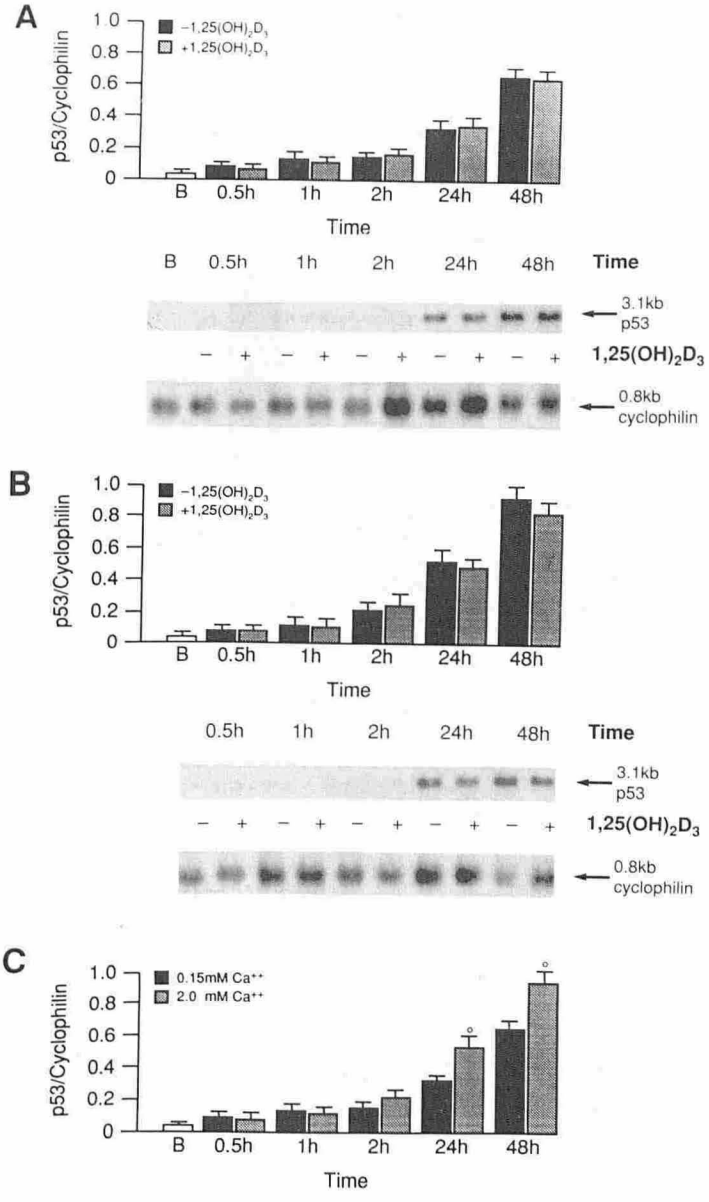

Figure 5. Northern blot analysis of p53 mRNA in normal human keratinocyte extracts. Filters were hybridized as described in *Materials and Methods* with a ^{32}P-labeled p53 probe and a ^{32}P-labeled cyclophilin probe as a control for RNA loading. The upper panels represent the densitometric ratios of p53/cyclophilin mRNA seen in lower panels. The *arrows* represent the 3.1 kb p53 message and the 0.8 kb cyclophilin message. Influence of $1,25(OH)_2D_3$ in 0.15 mM Ca^{++} *(A)* or 2.0 mM Ca^{++} *(B)*, and the influence of Ca^{++} *(C)* on the time course of p53 mRNA. Each lane contained 10 μg of total cellular RNA. The results are representative of three separate experiments. The ratios are expressed as mean ± SEM of three different experiments. *Asterisks* represent significant differences from corresponding incubations in absence of $1,25(OH)_2D_3$ and *circles* in the presence of 2.0 mM calcium.

We also showed that the EGF effects on keratinocyte cell growth were preceded by a rapid and transient increase in *c-fos* mRNA levels. This is consistent with the effects of various growth stimulants on the proto-oncogenes, *c-myc* and *c-fos*, in these and other cell types [45,46], suggesting that these proto-oncogenes may play an important role in the growth and differentiation of a variety of cells [4,47]. Earlier studies from this laboratory showed that $1,25(OH)_2D_3$ inhibited *c-myc* expression in primary cultures of parathyroid cells [48], which preceded the growth inhibition observed with $1,25(OH)_2D_3$. More recently, $1,25(OH)_2D_3$ was shown to inhibit *c-myc* proto-oncogene expression in normal human keratinocytes [10]. Furthermore, inhibition of *c-myc* expression by addition of antisense oligonucleotides to the culture medium of HeLa cells produced a growth arrest in G_0/G_1 [49], implying a causal relationship between *c-myc* expression and cellular growth. However, the relationship between *c-fos* and cellular differentiation was less clear. *fos* is known to modulate gene transcription by association with another proto-oncogene product, *jun*, to form a heterodimer or AP_1 protein complex [50]. Earlier studies showed that the *c-fos* gene was stimulated by factors that strongly influence cellular differentiation in PC12 cells, such as nerve growth factor [5]. It was also shown that $1,25(OH)_2D_3$ produced a sustained elevation of *c-fos* mRNA in HL 60 leukemic cells [51], associated with their monocytic differentiation. However, a direct effect of *fos* on cellular differentiation was not yet demonstrated. In the present

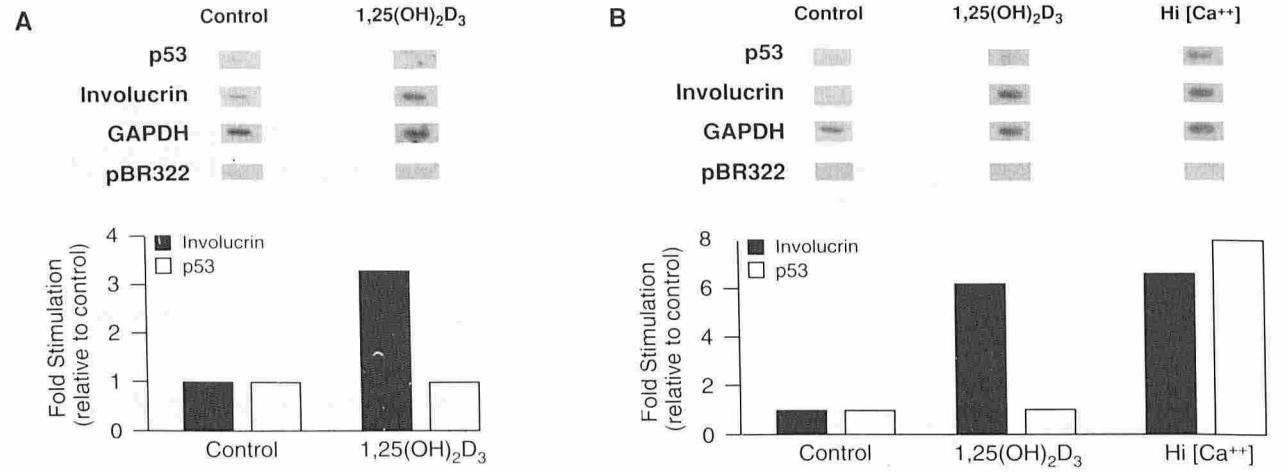

Figure 6. $1,25(OH)_2D_3$ and Ca^{++} effects on involucrin and p53 gene transcription. Nuclear run-on assays were performed as described in *Materials and Methods*. ^{32}P-labeled run-on transcripts were prepared from nuclei isolated from cells cultured for 2 h *(A)* and 24 h *(B)* in low (0.15 mM) or high (Hi) (2.0 mM) Ca^{++} concentrations in the presence or absence of 10^{-8} M $1,25(OH)_2D_3$. Autoradiographs of a representative experiment are shown in the upper panel (p53, involucrin, GAPDH, and plasmid pBR322 with no insert to assess non-specific binding). The relative transcription rate for involucrin and p53 over GAPDH are shown in the lower panel. Results are representative of three different experiments.

study, addition of $1,25(OH)_2D_3$ to growth factor stimulated keratinocytes increased *c-fos* expression; this effect was sustained over a long period. A sharp rise in *fos* expression was seen as early as 60 min after addition of growth factor but the combination of $1,25(OH)_2D_3$ and growth factors were clearly additive on *c-fos* mRNA levels. In contrast, addition of Ca^{++} did not significantly change *fos* mRNA levels whether $1,25(OH)_2D_3$ was present or not. The absence of effect of calcium on *c-fos* mRNA was consistent with previous observations in mouse [52] and human keratinocytes [38] and suggested that $1,25(OH)_2D_3$ could exert a specific effect on the expression of a proto-oncogene frequently associated with cellular differentiation. It is tempting to conclude that these results are mechanistically related. But further studies, such as those using antisense oligonucleotides [49,53] should be used to assess the specificity of *c-fos* induced promotion of cell differentiation by $1,25(OH)_2D_3$ in normal human keratinocytes.

Finally we examined the influence of EGF, calcium, and $1,25(OH)_2D_3$ on expression of p53 in keratinocytes. Recent evidence suggests that wild type p53 functions as a tumor suppressor gene [11,54–56] and that its inactivation by mutation or deletion is associated with neoplastic growth. However, p53 may also play an important role in growth of normal cells, as suggested by its regulated expression in a number of normal cells [57–59]. Previous studies showed that wild type p53 is necessary for the mitogenic response of lymphocytes [13] and 3T3 cells [14,15]. p53 subcellular localization varies throughout the cell cycle, accumulating in the nucleus following the initial step of DNA synthesis, around the beginning of the S phase; re-accumulating in the cytoplasm during the resting phase. This suggests that the protein is spatially regulated during the cell cycle [60]. In the present study we observed that p53 mRNA is rapidly induced by EGF, which is a necessary mitogen for the growth of keratinocytes in serum free conditions [61]. This close cell-cycle association also supports a role for p53 in normal keratinocyte cell growth. Because p53 acts normally as a growth suppressor, its induction by mitogenic stimuli, that we and others have observed, may represent a critical step in the control of cell growth to counteract the effect of mitogenic stimuli. However, the precise mechanism by which EGF modulates p53 gene transcription remains elusive and requires further studies. We also observed that p53 mRNA levels were sustained long after EGF stimulation; in sharp contrast *c-fos* mRNA stimulation by mitogens returns to basal levels quickly after its early peak. Recent data indicate that the p53 gene product may mediate the repression of *c-fos* mRNA, possibly acting as a transcriptional regulator of the *c-fos* promoter [62]. The temporal pattern of *c-fos* and p53 mRNA observed in our study would be consistent with such a mechanism. Finally, we observed that raising medium Ca^{++} from 0.15 to 2.0 mM significantly enhanced both steady-state p53 mRNA levels and the rate of p53 gene transcription, indicating that p53 is at least in part under the transcriptional control of Ca^{++}. These results are highly suggestive of a role for p53 in calcium mediated growth inhibition and induction of differentiation in keratinocytes but will require more direct evidence, such as the inhibition of intra-cellular p53 content by microinjection of p53 monoclonal antibodies [14] or introduction of plasmids coding for antisense p53 [63].

Our data nevertheless clearly suggest that $1,25(OH)_2D_3$ and calcium, which are essential in the control of growth and differentiation of normal human keratinocytes, act through a network of specific genes that are thought to be important in the control of the cell cycle. These co-ordinated responses, under the influence of $1,25(OH)_2D_3$ and calcium, seem to simultaneously control entry into the S phase of the cell cycle and at the same time trigger signals to initiate the differentiation process. Further studies aimed at blocking and/or activating *fos* and/or p53 genes should help clarify whether $1,25(OH)_2D_3$ and calcium affect growth and differentiation by a direct modulation of the expression of these cell-cycle-associated genes.

We would like to thank Mrs. D. Allen, G. Chang, K. Patel and J. Marshall for their excellent secretarial assistance, V. Papavasiliou for excellent technical assistance, Dr. M. Ratcliffe and Dr. J. Henderson for their expert assistance, Dr. M. Uskokovic for providing 1,25 dihydroxyvitamin D_3 and Dr. C. Bastomsky for kindly reviewing this manuscript.

This work was supported by grant MT-10839 (R. Kremer) from the Medical Research Council of Canada.

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