We have examined the effect of 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$) on mitogen-stimulated growth and on c-myc proto-oncogene expression in a keratinocyte model of tumor progression. A dose-dependent inhibition of growth by 1,25-(OH)$_2$D$_3$ was demonstrated in both established (HPK1A) and malignant (HPK1A-ras) cells. However, this inhibition was observed with the addition of 1,25-(OH)$_2$D$_3$ at a higher concentration in HPK1A-ras cells than in HPK1A cells. Cell cycle analysis revealed a blockade of the normal progression of the cell cycle from G$_0$ to S phase in the presence of 1,25-(OH)$_2$D$_3$. A higher concentration of 1,25-(OH)$_2$D$_3$ was required in HPK1A-ras cells to overcome the mitogen-stimulated progression into S phase, when compared with HPK1A cells. Analysis of c-myc messenger RNA revealed a strong inhibition of its expression at early time points with higher concentrations of 1,25-(OH)$_2$D$_3$ being required to obtain an inhibition in HPK1A-ras cells similar to that obtained in HPK1A cells. 1,25-(OH)$_2$D$_3$ receptor characterization by sucrose gradient analysis and equilibrium binding demonstrated the presence of a single 3.7 S protein with similar receptor numbers and affinity in both cell lines. These observations therefore demonstrate that an alteration of the growth inhibitory response to 1,25-(OH)$_2$D$_3$ occurs when keratinocytes acquire the malignant phenotype and suggest that the alteration lies beyond the interaction of the ligand with its receptor. In addition, relative resistance to 1,25-(OH)$_2$D$_3$ was also observed in the expression of the cell-cycle associated oncogene c-myc. These studies may therefore have important implications in vivo in the development and growth of epithelial cell cancers.

Carcinogenesis is a multistep process (1), and epidemiological studies have suggested that five or six independent steps are required for acquisition of the malignant phenotype (2). One of the mechanisms that has been implicated in neoplastic development is the cooperative action of two or more oncogenes (3) which are thought to act in a positive way to control cell growth in response to known mitogenic factors (4). However, equally important to the tumor development process may be negative regulators of cell growth which oppose the action of known growth factors. 1,25-Dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$) has been identified recently as an important factor controlling the growth of HL-60 human promyelocytic leukemic cells and their differentiation into the monocytic cell type (5, 6). This steroid has also been shown to have antiproliferative capabilities in normal keratinocytes (7, 8). Specific receptors for 1,25-(OH)$_2$D$_3$ have been demonstrated in both normal and malignant murine keratinocytes (9) which are thought to mediate the biological effects of the hormone. In addition to its inhibitory action on growth, previous studies have demonstrated an altered expression of specific oncogenes, such as c-myc, in response to 1,25-(OH)$_2$D$_3$ (10, 11). In normal human keratinocytes alterations in growth elicited by 1,25-(OH)$_2$D$_3$ were shown to be accompanied by a rapid inhibition of c-myc (13) expression. In the present study we have examined the effects of 1,25-(OH)$_2$D$_3$ on growth and c-myc expression in a human keratinocyte model of tumor cell progression (14, 15). In this model primary human foreskin keratinocytes were established as an immortalized cell line after transfection with human papillomavirus type 16 and subsequently transformed by an activated ras oncogene. Using this model system we have studied the effects of factors shown previously to modulate the growth of normal human keratinocytes, such as epidermal growth factor (EGF), fetal bovine serum (FBS), and 1,25-(OH)$_2$D$_3$.

**MATERIALS AND METHODS**

**Culture of Human Keratinocyte Cell Lines**—The HPK1A cell line was established from normal human keratinocytes by stable transfection with human papillomavirus type 16 (14). Despite acquiring an indefinite life span in culture these cells retain differentiation properties characteristic of normal keratinocytes (16) and are nontumorigenic when injected into nude mice (Table I). These immortalized cells were subsequently transformed into the malignant HPK1A-ras cell line after transfection with a plasmid carrying an activated H-ras oncogene (15). In addition to forming colonies in soft agar the malignant HPK1A-ras cells produce invasive squamous cell carcinoma when transplanted into nude mice (Table I). Both immortalized (HPK1A) and malignant (HPK1A-ras) cell lines were seeded and grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% FBS (GIBCO) and passaged once or twice weekly.

**XTT-Microculture Tetrazolium Assay for Cell Growth**—This assay, performed as described previously (17), assesses cellular growth on the basis of the intensity of a colorimetric reaction resulting from the reduction of a tetrazolium reagent (XTT), Polysciences, Warrington.

1. The abbreviations used are: 1,25-(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; EGF, epidermal growth factor; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; VDR, vitamin D receptor; VDRE, vitamin D-responsive element.

2. W. Gulliver, J. Henderson, and R. Kremer, submitted for publication.
PA to a soluble formazan salt by growing cells, absorbance being directly proportional to cell density. Linearity of the reaction over a wide range of cell numbers was assessed in preliminary experiments. Briefly, cells were seeded at 2 × 10^3 cells/100 μl into 96-well microtiter plates in DMEM containing 10% FBS. After 24 h in basal conditions (no growth factors), medium was removed and replaced with fresh DMEM supplemented with 10 ng/ml EGF (Sigma) and varying concentrations of 1,25-(OH)_{2}D_{3} (a kind gift of Dr. M. Uskokovic, Hoffman LaRoche, Nutley, NJ). After 24 h, 5 ml of XTT was made up to 1 ml, mixed with 1.53 mg/ml of phenylmethylsulfate (1.53 mg/ml in phosphate-buffered saline (Sigma)), and 50 μl of this mixture was added to each well. Absorbance was measured using a Bio-Rad microplate reader. Background absorbance was subtracted at each point using a reference absorbance of 0.650 nm.

**TABLE 1**

| Human keratinocyte model of tumor progression | Normal | HPV-16 | Immortalized | Transformed |
|---------------------------------------------|--------|--------|--------------|-------------|
| NHK                                         | HPKIA  | HPV16+ | HPKIA-ras    |
| 1. Stratification^a                        | +++    | −      | −            | +++         |
| 2. Differentiation^a                       | ++     | ++     | +++          | +++         |
| 3. Passages in culture                      | +      | +      | +            |              |
| 4. Tumorigenicity^d                        | −      | −      | +++          |              |

^a Human papillomavirus type 16.

^d Ref. 14.

^Ref. 16.

^Ref. 15.

**RESULTS**

**Effects of 1,25-(OH)_{2}D_{3} on Cell Growth**—The addition of increasing concentrations of 1,25-(OH)_{2}D_{3} to the culture medium produced a dose-dependent inhibition of cell growth in cultures of both established (HPKIA) and malignant (HPKIA-ras) keratinocytes (Fig. 1). Formazan production (Fig. 1A), \[^{3}H\]thymidine incorporation (Fig. 1B), and HPKIA-ras cells. In addition, the degree of inhibition at any one dose of 1,25-(OH)_{2}D_{3} was greater in HPKIA than in HPKIA-ras cultures. The time course of inhibition of \[^{3}H\]thymidine incorporation was also different in the two cell lines (Table II). The established (HPKIA) cells demonstrated a progressive and sustained response to 10^{-7} M 1,25-(OH)_{2}D_{3} over 72 h compared with the transient effect noted in the malignant (HPKIA-ras) cells.

**Effect of 1,25-(OH)_{2}D_{3} on the Cell Cycle**—Using flow cytometry, we next examined the effect of 1,25-(OH)_{2}D_{3} on the progression of HPKIA and HPKIA-ras cells through the cell cycle (Fig. 2). In the quiescent state the majority of cells were in the G0/G1 phase in both HPKIA (panel A) and HPKIA-ras (panel B) cultures. The addition of 10 ng/ml EGF to the culture medium for 24 h resulted in a shift into S phase in both cell lines. The addition of 10^{-7} M 1,25-(OH)_{2}D_{3} along with 10 ng/ml EGF produced an increase in the percentage of cells in G0/G1 phase, consistent with a block in cell cycle progression.
with the EGF inhibited the shift into S phase in HPK1A cells but had little effect in HPK1A-ras cells. However, the addition of \(10^{-6} \text{M} 1,25-(\text{OH})_2\text{D}_3\) with EGF resulted in an accumulation of cells in G0/G1 and a reduction of cells in S phase in both HPK1A and HPK1A-ras cultures.

**Dot Blot Analysis**—Total cellular RNA extracted from equal numbers of HPK1A and HPK1A-ras cells at timed intervals, after stimulation with 10% FBS, revealed a rapid and transient induction of c-myc mRNA in both cell lines. When expressed as fold stimulation above basal, the induction was greater in HPK1A than in HPK1A-ras cultures (Fig. 3). Dose-dependent decreases in this FBS-stimulated activity in the absence and presence of \(1,25-(\text{OH})_2\text{D}_3\) were noted in both cell lines, although the inhibitory response was greater in the HPK1A than in HPK1A-ras cells (Fig. 4).

**Northern Analysis**—Northern analysis of total RNA removed from timed intervals from HPK1A and HPK1A-ras cells revealed one major transcript of 2.4 kilobases in both cell lines (Fig. 5). The addition of 10% FBS resulted in a stimulation of c-myc mRNA expression which was greater in HPK1A (panel A) than in HPK1A-ras (panel B). Although

**TABLE II**

| Time | [\(\text{HU}\)]Thymidine uptake |
|------|-------------------------------|
|       | HPK1A | HPK1A-ras |
| 24 h  | 72 ± 4* | 70 ± 1*   |
| 48 h  | 55 ± 2* | 64 ± 4*   |
| 72 h  | 47 ± 2* | 88 ± 2*   |

* Significant difference from control.

After 24 h in basal conditions (no growth factors), fresh medium containing EGF (10 ng/ml) and \(10^{-7} \text{M} 1,25-(\text{OH})_2\text{D}_3\) was added to cultures of HPK1A and HPK1A-ras cells. [\(\text{HU}\)]Thymidine uptake was assessed at timed intervals, and results are expressed as a percentage of EGF-stimulated activity. Each value represents the mean ± S.E. of six determinations and is representative of three separate experiments.

**Fig. 1.** Effect of 1,25-(OH)\(_2\)D\(_3\) on EGF-stimulated cell growth in HPK1A and HPK1A-ras cells. After 24 h in basal conditions (no growth factors), fresh DMEM containing EGF (10 ng/ml) without or with increasing concentrations of 1,25-(OH)\(_2\)D\(_3\) was added to cultures of HPK1A (O) and HPK1A-ras (A) cells at time 0. Panel A represents formazan production assessed at 24 h using the XTT assay described under "Materials and Methods." C represents control cultures incubated in the presence of 10 ng/ml EGF and in the absence of 1,25-(OH)\(_2\)D\(_3\). Panel B represents [\(\text{HU}\)]thymidine incorporation corrected for cell numbers and expressed as a percent of EGF-stimulated activity (100%). Panel C represents cell numbers assessed at 96 h and expressed as percent of EGF-stimulated cell numbers (100%). Each value represents the mean ± S.E. of four to six determinations and is representative of three different experiments. Asterisks indicate a significant difference from control values (EGF-stimulated activity), and open circles indicate a significant difference between HPK1A and HPK1A-ras cells at the 1,25-(OH)\(_2\)D\(_3\) concentration indicated.

**Fig. 2.** Cell cycle analysis of EGF-stimulated HPK1A and HPK1A-ras cells in the absence and presence of 1,25-(OH)\(_2\)D\(_3\). After 24 h in basal conditions (no growth factors) (black bars), fresh DMEM supplemented with EGF (10 ng/ml) (white bars), or EGF plus 1,25-(OH)\(_2\)D\(_3\) at \(10^{-8} \text{M}\) (light gray bars) or \(10^{-7} \text{M}\) (dark gray bars) was added to cultures of HPK1A (panel A) and HPK1A-ras (panel B) cells at time 0. 24 h later cells were trypsinized and analyzed by flow cytometry as described under "Materials and Methods." Results are expressed as percentage of cells distributed in G0/G1 phase (left panel) and S phase (right panel) of the cell cycle. Each bar represents the mean ± S.E. of four determinations and is representative of duplicate experiments. Asterisks indicate a significant difference from EGF stimulation alone.
**1,25-Dihydroxyvitamin D₃ Resistance**

![Graph](image)

**Fig. 3.** Dot blot analysis of c-myc mRNA in HPK1A and HPK1A-ras keratinocytes. After 24 h in basal conditions (time 0), fresh DMEM supplemented with 10% FBS was added to cultures of HPK1A (○) and HPK1A-ras (△) cells at time 0. Total cellular RNA was extracted from equal numbers of cells removed at timed intervals as described under “Materials and Methods.” Nytran filters were probed with a Clal-EcoRI restriction fragment encoding exon III of the c-myc gene. Data are representative of three different experiments.

![Graph](image)

**Fig. 4.** Dot blot analysis of c-myc mRNA in HPK1A and HPK1A-ras cells treated with varying concentrations of 1,25-(OH)₂D₃. After 24 h in basal conditions, fresh DMEM containing 10% FBS without (○) or with increasing concentrations of 1,25-(OH)₂D₃ was added to cultures of HPK1A (□) and HPK1A-ras (■) keratinocytes. GTC extracts of equal numbers of cells collected at 6 h were subjected to dot blot analysis as described under “Materials and Methods.” Filters were hybridized with a Clal-EcoRI restriction fragment encoding exon III of the c-myc gene. Each bar represents the mean ± S.E. of triplicate determinations, and data are representative of three different experiments. Asterisks indicate a significant difference from incubations performed in the absence of 1,25-(OH)₂D₃ (○).

![Graph](image)

**Fig. 5.** Northern analysis of c-myc mRNA in HPK1A and HPK1A-ras keratinocytes. After 24 h in basal conditions (time 0), fresh DMEM supplemented with 10% FBS with (+) or without (−) 10⁻⁷ M 1,25-(OH)₂D₃ was added to cultures of HPK1A (panel A) and HPK1A-ras (panel B) cells at time 0. Total cellular RNA was extracted from cells removed at timed intervals as described under “Materials and Methods.” 10 μg of RNA/lane was electrophoresed on a 1.1% agarose-formaldehyde gel, blotted onto a Nytran filter, and probed as in Fig. 4. Ethidium bromide-stained gels demonstrated equivalent quantities of RNA loaded into all lanes, and cyclodiphenyl-probed filters showed little change from basal mRNA in all lanes.

![Graph](image)

**Fig. 6.** Sucrose density gradient analysis of [³H]1,25-(OH)₂D₃ binding to cytosolic receptors in HPK1A and HPK1A-ras cells. Binding studies were performed using 500 μg of cytosolic extract from HPK1A (panel A) and HPK1A-ras (panel B) cells were incubated with 10,000 cpm of [³H]1,25-(OH)₂D₃ in the absence (■) or presence (○) of 60 nM unlabeled 1,25-(OH)₂D₃, as described under “Materials and Methods.” Unbound hormone was removed with dextran-coated charcoal before centrifugation through linear 4–20% sucrose gradients. Sedimentation coefficients (3.7 S) were estimated using a ¹⁴C-labeled ovalbumin standard.

**DISCUSSION**

We have examined the effect of 1,25-(OH)₂D₃ on parameters of mitogen-stimulated cell growth and c-myc proto-oncogene expression in a keratinocyte model of tumor progression. Previous studies have identified 1,25-(OH)₂D₃ as a regulator of cell growth and differentiation in normal human keratinocytes (8, 13). Keratinocytes in culture have been shown to convert 25-hydroxyvitamin D₃ to its active metabolite, 1,25-(OH)₂D₃ (22), and cytosolic receptors for 1,25-(OH)₂D₃ have been identified in keratinocytes (9, 23, 24). This steroid, therefore, has the potential to inhibit growth and stimulate differentiation in epidermal cells in an autocrine manner.

In previous studies we demonstrated a sustained inhibition of mitogen-stimulated growth in normal human keratinocytes by 10⁻⁸ M 1,25-(OH)₂D₃. This inhibition was shown to be caused by a blockade in the transition from G1 → S, with an accumulation of cells in G₀/G₁ phase. In the present study, 10⁻⁸ M 1,25-(OH)₂D₃ was capable of inducing a similar sustained inhibition of mitogen-stimulated growth in the established HPK1A cell line, which was a result of blockage into S phase. However, the neoplastic HPK1A-ras keratinocytes demonstrated a transient growth inhibitory response which required 10–100-fold higher concentrations of 1,25-(OH)₂D₃ to achieve the same degree of inhibition as that seen in the
OH)3D3 has been shown to regulate c-myc gene expression. Suppression of c-myc mRNA was negatively regulated by 1,25-

duction of endogenous transforming growth factor-β (30), exogenous growth promoters could be a function of overpro-

phase into the actively dividing phase of the cell cycle. This proto-oncogene encodes a protein involved in the passage of cells from the resting to S phase. A similar resistance to the antimitogenic effect of 1,25-(OH)2D3 was observed in both cell lines with an early peak and a slow mRNA expression in response to stimulation by mitogens was observed in both cell lines with an early peak and a slow mRNA expression in response to stimulation by mitogens.

Having demonstrated that the response to 1,25-(OH)2D3 as a negative regulator of the cell cycle was diminished in the malignant keratinocytes, we then assessed and compared the levels of c-myc mRNA in response to serum and to 1,25-(OH)2D3. Recent studies were performed using 500 μg of cytosolic extract from HPK1A (panel A) and HPK1A-ras (panel B) cells in the absence or presence of increasing concentrations of unlabeled 1,25-(OH)2D3. Non-specific binding was assessed in the presence of 60 nM unlabeled 1,25-(OH)2D3. Dissociation constants (Kd) were calculated from the slopes of the lines. The number of receptor molecules (Bmax) are expressed as fmol/mg protein.

Established cells with 10−8 M 1,25-(OH)2D3. Analysis of the cell cycle of the malignant HPK1A-ras cells revealed a similar pattern of resistance, in which 10−8 M 1,25-(OH)2D3 was incapable of preventing the mitogen-stimulated passage of cells into S phase. A similar resistance to the antimitogenic effects of transforming growth factor-β, a known negative regulator of keratinocyte growth (25), has been reported previously in malignant keratinocytes (26) although the mechanism remains undefined.

Having demonstrated that the response to 1,25-(OH)2D3 as a negative regulator of the cell cycle was diminished in the malignant keratinocytes, we then assessed and compared the levels of c-myc mRNA in response to serum and to 1,25-(OH)2D3 in the HPK1A and HPK1A-ras cell lines. Recent studies have focused on a potential role for c-myc in the regulation of the cell cycle. This proto-oncogene encodes a highly conserved nuclear protein which is expressed in a tissue-specific manner in both fetus and adult (27). The c-myc gene product is thought to mediate a signal associated with cell division and appears to be required for normal cell growth (28). The rapid induction of c-myc after stimulation by agents such as growth factors and serum (4, 29) suggests a potential role for the protein in the passage of cells from the resting phase into the actively dividing phase of the cell cycle.

In the present study, a characteristic time course of c-myc mRNA expression in response to stimulation by mitogens was observed in both cell lines with an early peak and a slow decline over 24–48 h. However, maximum levels of induction were greater in HPK1A than in HPK1A-ras cells. The apparent independence, exhibited by the malignant cells, from exogenous growth promoters could be a function of overproduction of endogenous transforming growth factor-α (30), constitutively active EGF receptors (31), or altered positive responsive elements located in the promoter region of the c-myc gene (32).

In previous studies, we and others have shown that expression of c-myc mRNA was negatively regulated by 1,25-(OH)2D3 in several cell types (10–13, 33). In addition, 1,25-(OH)2D3 has been shown to regulate c-myc gene expression at the transcriptional level (33). In the present study, the immortalized HPK1A cells showed a strong inhibition of c-myc mRNA expression similar to that described previously in normal keratinocytes in response to 10−8 M 1,25-(OH)2D3. The neoplastic HPK1A-ras cells, on the other hand, responded to a 10-fold greater dose with minimal inhibition of serum-stimulated c-myc mRNA levels at early time points and with an apparent rebound at 24 h.

We have noted previously the same type of altered response at the mRNA level of another 1,25-(OH)2D3-responsive gene, the parathyroid hormone-related peptide (PTHrP) gene, in the HPK1A-ras keratinocytes (34), as compared with normal human keratinocytes (35). This relative resistance could occur at the level of receptor-ligand interaction as a function of altered intracellular metabolism of the steroid, altered receptor function or a constitutional absence, or decreased expression of the 1,25-(OH)2D3 receptor in the malignant cells. Soluble extracts from HPK1A and HPK1A-ras cells demonstrated similar 1,25-(OH)2D3 binding characteristics including receptor number, receptor affinity, and migration pattern of the receptor-ligand complex on a sucrose density gradient which were similar to those found in normal human keratinocytes (36). Analysis of medium conditioned by HPK1A and HPK1A-ras cells for 48 h in the presence of 10−7 1,25-(OH)2D3 showed no significant difference in the levels of the steroid (data not shown). Taken together, these observations suggest that the relative resistance to 1,25-(OH)2D3 demonstrated in the malignant keratinocytes in these studies lies somewhere beyond the interaction of the ligand with its cytosolic receptor.

The mechanism by which 1,25-(OH)2D3 suppresses gene activity remains elusive. One potential mechanism could involve direct suppression via binding of the 1,25-(OH)2D3-receptor complex to as yet unidentified responsive elements in target genes such as c-myc. An alternative mechanism could involve competition between the VDR and other tran-
sactivating factors which normally regulate transcriptional activity of that gene (37). Both positive and negative regulation of transcription by thyroid hormone receptor and retinoic acid receptor heterodimers has been demonstrated (38). In this context competition between positive regulators of c-myc expression, such as growth factors or serum, and the 1,25-(OH)2D3-receptor complex could be altered in the ras-transformed keratinocytes. Perhaps an excess of unidentified positive regulatory factors present in the HPK1A-ras cells confers a requirement for an increased concentration of 1,25-(OH)2D3 to form sufficient 1,25-(OH)2D3-receptor complexes to compete effectively for binding to the genetic regulatory element. Inasmuch as repression of c-myc transcriptional activity was measured in the present studies in the presence of serum this could prove to be a plausible hypothesis. Identification of a serum-responsive element, a vitamin D-responsive element (VDRE), and other regulatory factors as well as definition of their interactions with one another will be required to clarify this issue.

It has been suggested that binding of 1,25-(OH)2D3 to its receptor induces a conformational change allowing for recep-
tor phosphorylation (39). In addition, serine 51 of the VDR has been identified as an effective substrate for protein kinase C-β and the resulting phosphoprotein shown to be important to VDR-mediated transcription (40). An anomalous phosphorylation process in the ras-transformed cells could perhaps contribute to the 1,25-(OH)2D3 relative resistance demonstrated by these cells. Additionally a sequence between amino acids 102 and 112 outside of the DNA binding domain, in the VDR, has been postulated by Haussler et al. (37) to be necessary in the nuclear transfer of the receptor and could...
therefore constitute another potential site of inactivation in the ras-transformed cells. Alternative possibilities could encompass altered interactions between VDR and cis-acting VDREs and/or between VDR and other trans-activating factors necessary for transcriptional regulation (41) and/or for stabilization of the VDR/VDRE (12). Thus, the apparent transience of the inhibitory action of 1,25-(OH)2D3 in the malignant HPK1A-ras cells could be a function of an unstable VDR/VDRE association. Identification of both a consensus VDRE as well as accessory proteins involved in the interaction between the VDR and its target genes in these cells will be required to clarify these issues.

The preceding studies therefore demonstrate that resistance to an important negative regulator of keratinocyte cell growth is acquired in the passage from established to malignant phenotype. These findings may have important implications in vivo in the development and unrestrained growth of squamous carcinomas.

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