Regulation of Gene Expression by 1α,25-Dihydroxyvitamin D_3 and Its Analog EB1089 under Growth-Inhibitory Conditions in Squamous Carcinoma Cells

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Analogs of 1α,25-dihydroxyvitamin D_3 (1α, 25$(OH)_2D_3$) inhibit growth *in vitro* and *in vivo* of cells derived from a variety of tumors. Here, we examined the effects of 1α,25$(OH)_2D_3$ and its analog EB1089 on proliferation and target gene regulation of human head and neck squamous cell carcinoma (SCC) lines SCC4, SCC9, SCC15, and SCC25. A range of sensitivities to 1α,25$(OH)_2D_3$ and EB1089 was observed, from complete G_0/G_1 arrest of SCC25 cells to only 50% inhibition of SCC9 cell growth. All lines expressed similar levels of vitamin D_3 receptor (VDR) mRNA and protein, and no significant variation was observed in 1α,25$(OH)_2D_3$-dependent induction of the endogenous 24-hydroxylase gene, or of a transiently transfected 1α,25$(OH)_2D_3$-sensitive reporter gene. The antiproliferative effects of 1α,25$(OH)_2D_3$ and EB1089 in SCC25 cells were analyzed by screening more than 4,500 genes on two cDNA microarrays, yielding 38 up-regulated targets, including adhesion molecules, growth factors, kinases, and transcription factors. Genes encoding factors implicated in cell cycle regulation were induced, including the growth arrest and DNA damage gene, gadd45α, and the serum- and glucocorticoid-inducible kinase gene, sgk. Induction of GADD45α protein in EB1089-treated cells was confirmed by Western blotting. Moreover, while expression of proliferating cell nuclear antigen (PCNA) was reduced in EB1089-treated cells, coimmunoprecipitation studies revealed increased association between GADD45α and PCNA in treated cells, consistent with the capacity of GADD45α to stimulate DNA repair. While 1α,25$(OH)_2D_3$ and EB1089 modestly induced transcripts encoding the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$, no changes in protein levels were observed, indicating that $p21^{waf1/cip1}$ induction does not contribute to the antiproliferative effects of 1α,25$(OH)_2D_3$ and EB1089 in SCC cells. Finally, in partially resistant SCC9 cells, there was extensive loss of target gene regulation (10 of 10 genes tested), indicating that resistance arises from widespread loss of 1α,25$(OH)_2D_3$-dependent gene regulation in the presence of normal levels of functional VDRs. (Molecular Endocrinology 15: 1127–1139, 2001)

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INTRODUCTION

The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] modulates gene expression by binding to the vitamin D_3 receptor (VDR), which is a member of the nuclear receptor family of transcriptional regulators. 1α,25$(OH)_2D_3$-bound VDR heterodimerizes with retinoid X receptors (RXRs) and binds to specific DNA sequences in target genes known as vitamin D_3 response elements (VDREs) (1, 2). Apart from its well characterized role in calcium homeostasis (3), 1α,25$(OH)_2D_3$ also inhibits growth and stimulates differentiation of cancer cells derived from a variety of tissues, including breast, prostate,

colon, lung, endometrium, hematopoietic cells, and oral cavity (4–10). A side chain analog of $1\alpha,25(OH)_2D_3$, EB1089, caused apoptotic regression of MCF-7 breast carcinoma xenografts in nude mice (9), and animal studies and early clinical testing have shown that therapeutic doses of EB1089 can be tolerated without inducing hypercalcemia (10).

Analogs of $1\alpha,25(OH)_2D_3$ are potential candidates for chemoprevention of squamous cell carcinomas (SCCs) of the oral cavity, where formation of second primary carcinomas after surgical removal of tumors is a major concern (11, 12). Retinoids, such as 13-*cis* retinoic acid (13-*cis*-RA; isotretinoin) have been used clinically in SCC chemoprevention (13). 13-*cis*-RA functions by binding to retinoic acid receptors (RARs), which, like the VDR, are nuclear receptors and function as heterodimers with RXRs (1). However, SCC progression is associated with reduced expression of RARs, particularly RARs β and γ, loss of retinoid-regulated differentiation markers, and resistance to the antiproliferative effects of retinoids (14–19).

Here, we have examined the effect of $1\alpha,25(OH)_2D_3$ and EB1089 on proliferation and target gene regulation of four human SCC lines, SCC4, SCC9, SCC15, and SCC25, which were derived from the floor of mouth/base of tongue lesions (14). SCC25 cells express near normal levels of RARs β and γ and retain retinoid regulation of keratin-19 (K-19) gene expression, whereas SCC4, SCC9, and SCC15 cells express reduced levels of RARγ, no RARβ, and have lost regulated K-19 expression (14). The SCC lines display differing sensitivities to $1\alpha,25(OH)_2D_3$ and EB1089. SCC25 cell growth was completely blocked by $1\alpha,25(OH)_2D_3$ and EB1089, while the other lines were partially resistant. We have identified 38 $1\alpha,25(OH)_2D_3$ target genes in SCC25 cells, which encode several components of signal transduction pathways. Our results indicate that the antiproliferative effects of $1\alpha,25(OH)_2D_3$ and its analogs are mediated by multiple downstream components. Moreover, resistance to $1\alpha,25(OH)_2D_3$ in SCC9 cells was accompanied by widespread loss of target gene regulation in spite of normal levels of functional VDRs.

RESULTS

Effect of $1\alpha,25(OH)_2D_3$ and EB1089 on Growth of SCC Lines

The growth-inhibitory effects of $1\alpha,25(OH)_2D_3$, EB1089, and 13-*cis*-RA were evaluated in human lines SCC4, SCC9, SCC15, and SCC25, derived from SCCs of the oral cavity. The four lines displayed different sensitivities to $1\alpha,25(OH)_2D_3$ or EB1089 (Fig. 1). Over 10 days, SCC25 cell growth was completely inhibited by 100 nM $1\alpha,25(OH)_2D_3$, and 1–100 nM EB1089 (Fig. 1, A and B), while SCC4, SCC9, and SCC15 cells displayed partial resistance to both compounds (Fig. 1, D, E, G, H, J, and K). Similarly, SCC25 cell growth was strongly inhibited by 13-*cis*-RA (100 nM; Fig. 1C), whereas growth of SCC4, SCC9, and SCC15 cells was partially resistant (Fig. 1, F, I, and L). Flow cytometric analysis showed that treatment of SCC25 cells with 100 nM EB1089 for 72 h reduced the number of cells in S phase by 2.5-fold and significantly increased the percentage in G_0/G_1 (Fig. 2A). No evidence for DNA fragmentation was observed by terminal deoxynucleotidyltransferase dUTP-biotin nick end-labeling (TUNEL) assays under these conditions or over extended periods (Fig. 2B).

Resistance to $1\alpha,25(OH)_2D_3$ in SCC4, SCC9, and SCC15 cells Is Not Accompanied by Loss of Expression of Functional VDR

Given that resistance to 13-*cis*-RA correlated with lost or reduced expression of RARs β and γ, respectively (14), it was of interest to examine the levels of functional VDR in SCC cells. Northern and Western blots showed that VDR transcript and protein levels were essentially identical in all four lines (Fig. 3, A and B). Similarly, no evidence was found for loss of expression of the two major RXRs expressed in SCC, RXRα and RXRβ (data not shown). VDR function was tested by transient transfection of a $1\alpha,25(OH)_2D_3$-sensitive reporter-promoter plasmid containing a bacterial lacZ gene under control of a synthetic promoter containing three VDREs (20). High levels of $1\alpha,25(OH)_2D_3$-inducible β-galactosidase activity were detected in all cell extracts (Fig. 4A), suggesting that the lines expressed similar levels of functional VDRs. Both $1\alpha,25(OH)_2D_3$ and EB1089 induced similar levels of expression of the endogenous 24-hydroxylase (24-OHase) gene (Fig. 4B), whose promoter contains VDREs (21). Moreover, EB1089 induced 24-OHase expression with essentially identical potencies in $1\alpha,25(OH)_2D_3$-sensitive SCC25 cells and the partially resistant lines SCC4 and SCC9 (Fig. 4C). Taken together, the results of Figs. 3 and 4 suggest that resistance to $1\alpha,25(OH)_2D_3$ does not arise through loss of expression of functional VDRs.

Effects of $1\alpha,25(OH)_2D_3$ and EB1089 on Cell Cycle Regulators in SCC25 Cells

We were interested in analyzing the mechanisms underlying the antiproliferative effects of $1\alpha,25(OH)_2D_3$ and EB1089 in SCC25 cells. Previous work has shown that $1\alpha,25(OH)_2D_3$ rapidly (4 h) and strongly stimulated expression of the cyclin-dependent kinase inhibitor genes $p21^{waf1/cip1}$ and $p27^{kip1}$ in myeloid leukemia cells under conditions where it induced differentiation and inhibited cell growth (4, 22). However, the magnitude of the effect of $1\alpha,25(OH)_2D_3$ on $p21^{waf1/cip1}$ expression varies widely in different cell lines (4, 6, 22–24). We found that $1\alpha,25(OH)_2D_3$- or EB1089-dependent induction of $p21^{waf1/cip1}$ transcripts in SCC25 cells was gradual and modest (Fig. 5A and data not shown), whereas no effect was observed on expression of $p27^{kip1}$ or p53 mRNA levels (Fig. 5B). The modest effect of $1\alpha,25(OH)_2D_3$ and EB1089 on $p21^{waf1/cip1}$ mRNA levels did not give rise to significant changes in $p21^{waf1/cip1}$ protein, however. In addition, no effect of $1\alpha,25(OH)_2D_3$ or EB1089 was observed on $p27^{kip1}$ protein levels (Fig. 5C).

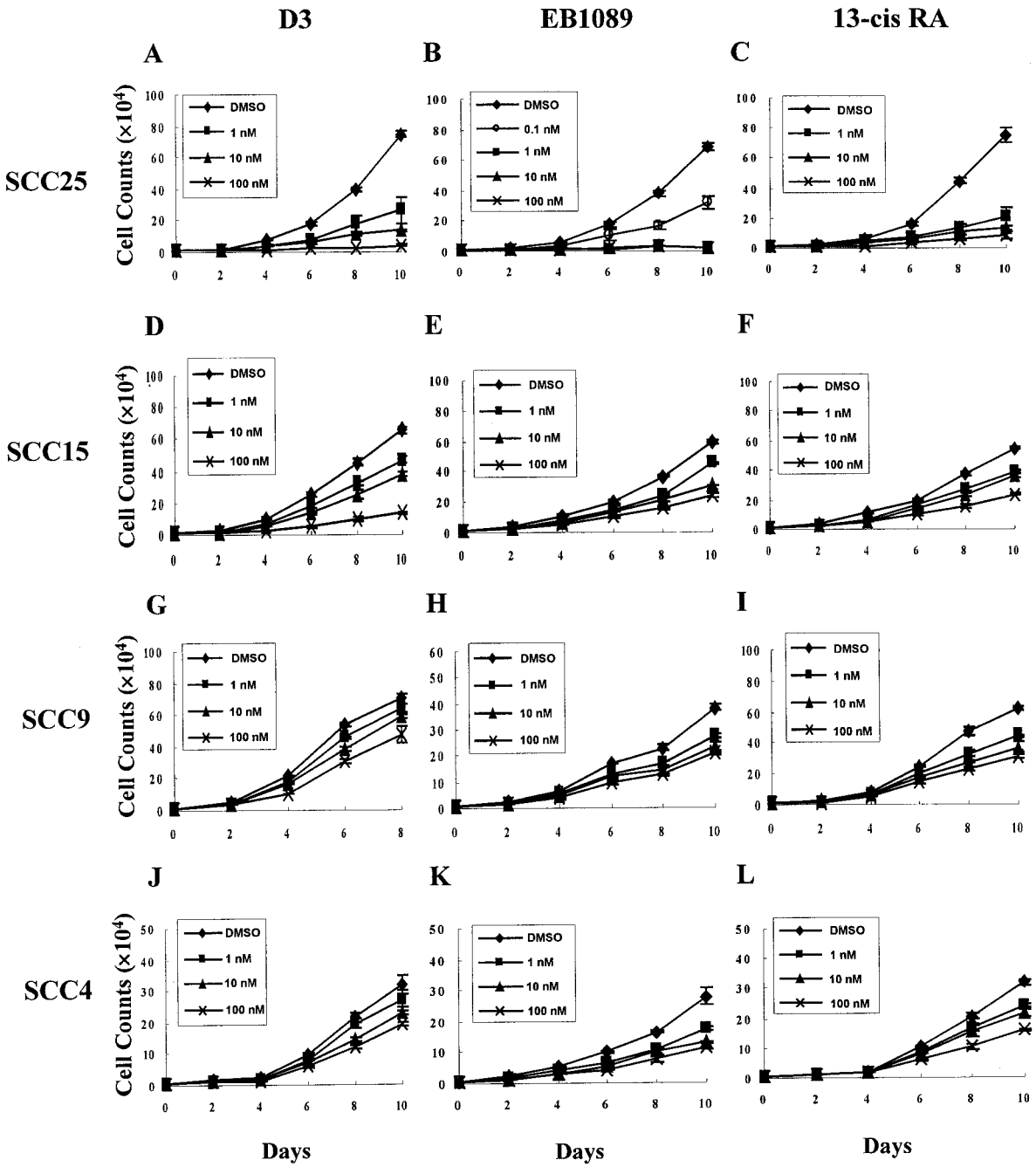

Fig. 1. Dose-Dependent Effects of 1α,25(OH)$_2$D$_3$, EB1089, and 13-*cis*-RA on Proliferation of SCC Lines in Culture

SCC lines were treated with 1, 10, or 100 nM 1α,25(OH)$_2$D$_3$ (A, D, G, and J), 0.1, 1, 10, or 100 nM EB1089 (B, E, H, and K), and 1, 10, or 100 nM 13-*cis*-RA (C, F, I, and L). Media were changed and fresh ligand added every 2 days over the 10-day period of the experiment. Each *point* represents the result obtained from triplicate wells (see *Materials and Methods* for details).

Identification of Target Genes of 1α,25(OH)$_2$D$_3$ and EB1089 by Screening of cDNA Microarrays

We screened cDNA microarrays for novel target genes of 1α,25(OH)$_2$D$_3$ and EB1089 in SCC25 cells to identify factors mediating their antiproliferative effects. More than 4,500 genes on two different gene arrays [Atlas array, 588 genes; (CLONTECH Laboratories, Inc., Palo Alto, CA); Named Genes filter, 4,000+ genes (Research Genetics, Inc., Huntsville, AL)] were screened with probes derived from vehicle-treated cells or cells treated with EB1089 for 24 h. Previous work has shown that there is considerable variation in gene expression levels associated with screening gene arrays (25–28). Arrays were therefore screened multiple times, and only reproducibly regulated genes were retained. Two rounds of screening of Atlas arrays yielded 10 candidate genes, of which 6 were revealed

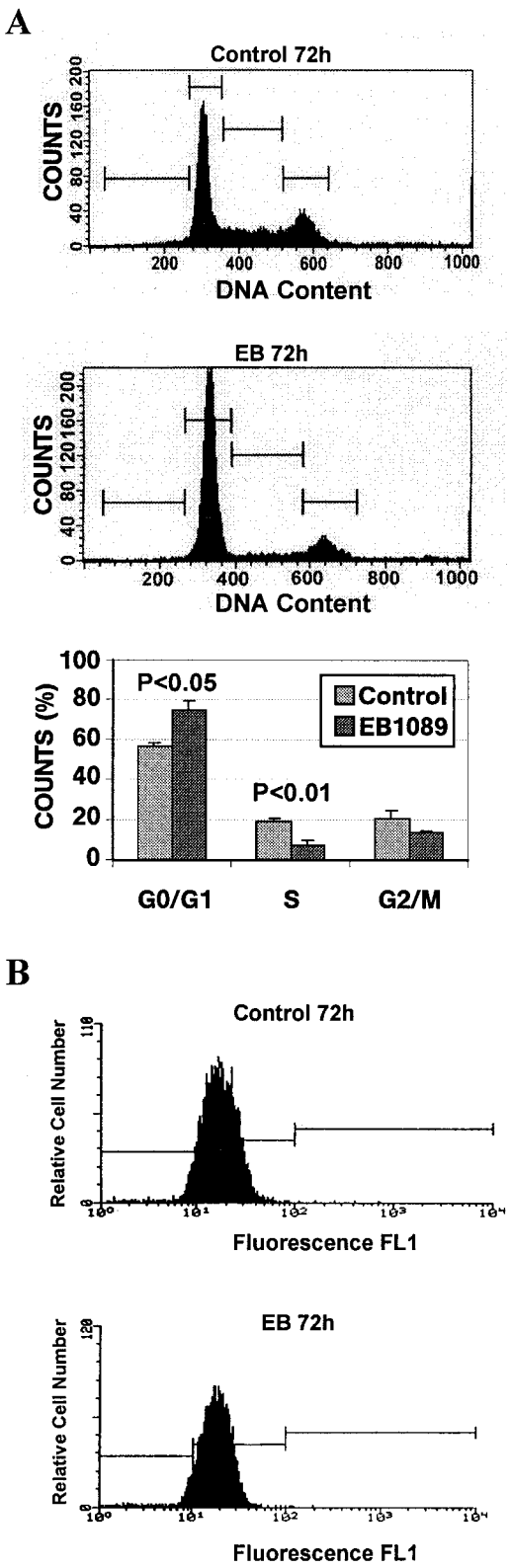

Fig. 2. Flow Cytometric Analysis and TUNEL Assay of Control or EB1089-Treated SCC25 Cells

A, SCC25 cells were treated with vehicle (SCC25 cont) or EB1089 (SCC25 EB) for 72 h. A histogram of fractions of cells in G_0/G_1, S, or G_2 from three independent experiments is presented. Statistical significance was determined using Student's *t* test. B, Representative histograms of three experiments assessing 3′-OH end labeling characteristic of apoptotic cells (TUNEL assay). Control cells and cells treated for 72 h with 100 nM EB1089 display minimal DNA fragmentation.

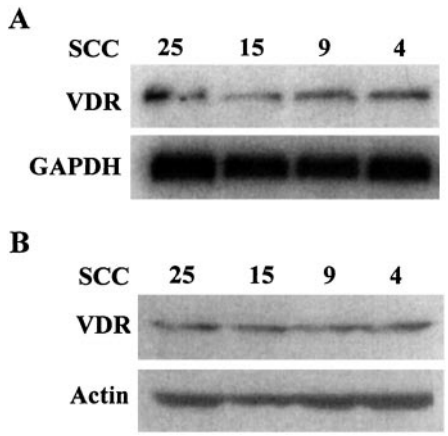

Fig. 3. Expression of VDR Transcripts and Protein in SCC Lines

A, Northern analyses are presented of transcripts encoding the VDR in SCC lines, along with GAPDH controls. B, Western blots are presented of VDR and β-actin protein levels in SCC lines.

by Northern blotting to be regulated by $1\alpha,25(OH)_2D_3$ and EB1089 (Fig. 6A, and data not shown; Table 1). In addition to $p21^{waf1/cip1}$ (not shown), these included novel target genes amphiregulin, a member of the epidermal growth factor family, the transcription factor fos-related antigen-1 (fra-1), the growth arrest and DNA damage (gadd45α) gene, and integrin α7B. We also found that the vascular endothelial growth factor (VEGF), which has been shown to be a $1\alpha,25(OH)_2D_3$ target gene in osteoblast-like cells (29, 30), was regulated by EB1089 in SCC25 cells.

Initial analysis of Research Genetics, Inc. gene filters screened with duplicate preparations of probe from vehicle-treated cells revealed a substantial number of differentially expressed genes (data not shown), which likely corresponded to random fluctuations in gene expression observed in expression profiling (25–28). Therefore, filters were screened three times each with probe from independent preparations of vehicle- and EB1089-treated cells, generating nine sets of cross-comparisons. Genes that were reproducibly regulated at least 1.5-fold in all comparisons were conserved. This yielded 32 additional up-regulated genes representing several different classes of proteins (Table 1). Screening under these conditions did not reveal any reproducibly down-regulated genes. Up-regulated genes included calmodulin, which has previously been shown to be a $1\alpha,25(OH)_2D_3$ target gene (31). Northern blotting, used to further test expression of 10 of these genes, revealed EB1089-stimulated expression in all

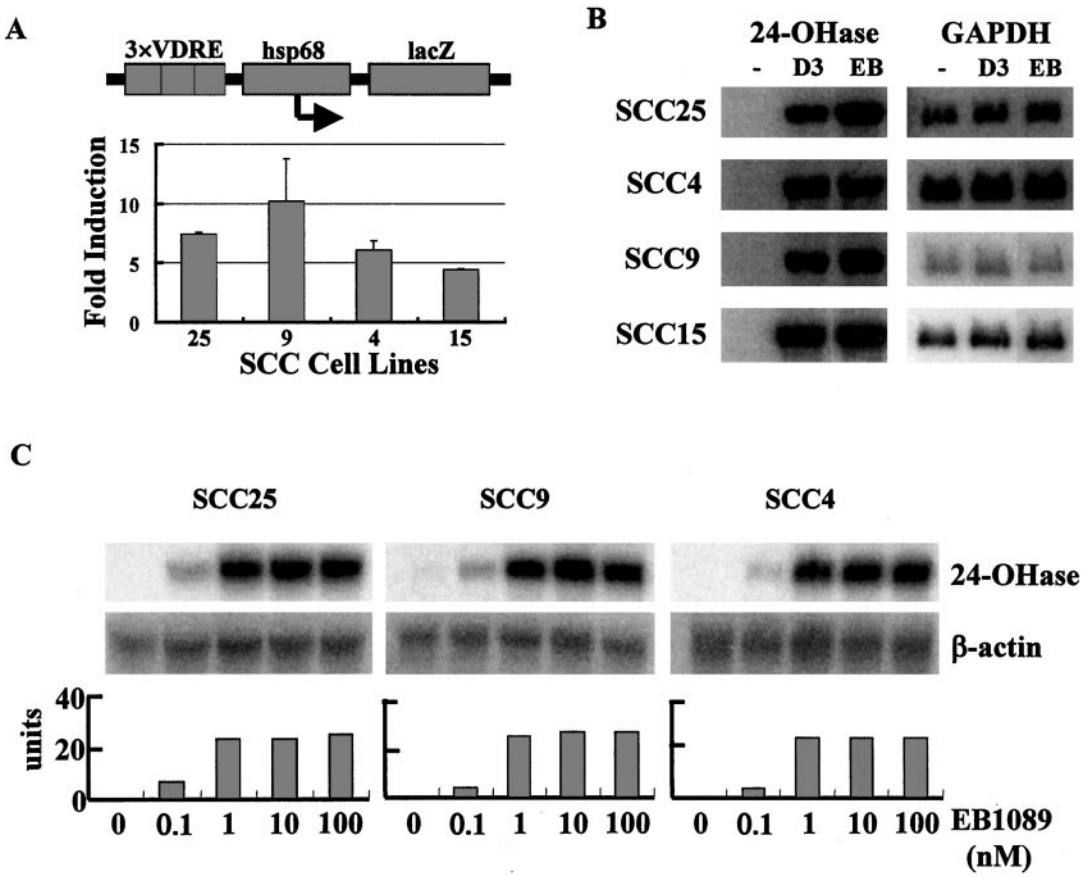

Fig. 4. Assessment of VDR Function in SCC Lines

A, Cells were transfected with the 1α,25(OH)$_2$D$_3$-sensitive reporter plasmid VDRE3-hsp68-lacZ and treated with vehicle or 10 nM 1α,25(OH)$_2$D$_3$ for 24 h (see *Materials and Methods* for details). Data are presented as fold induction of lacZ expression observed in the presence of 1α,25(OH)$_2$D$_3$. B, Induction of endogenous 24-OHase gene expression by 1α,25(OH)$_2$D$_3$ or EB1089 in SCC lines. Northern blots of total RNA extracted from cells treated for 24 h with vehicle (−), 1α,25(OH)$_2$D$_3$-, or EB1089-treated cells are presented. C, Dose-dependence of 24-hydroxylase induction in 1α,25(OH)$_2$D$_3$-sensitive SCC25 cells and partially resistant SCC4 and SCC9 cells. Northern blots of 24-OHase and β-actin controls are presented above, along with the normalized results of densitometric scanning of the 24-OHase blots below.

cases (Fig. 6B), indicating that the data in Table 1 are highly reliable. Most of the genes retained from phosphorimager analysis of the Research Genetics, Inc. arrays were up-regulated 2- to 4-fold (Table 1). This range of induction agrees well with that of up-regulated targets identified in a similar screen of thyroid hormone-regulated genes (33).

Broad but Selective Loss of Target Gene Expression in 1α,25(OH)$_2$D$_3$-Resistant SCC Lines

Given the resistance of SCC9 cells to the inhibitory effects of 1α,25(OH)$_2$D$_3$ and EB1089, we analyzed the regulation of target genes in these cells. Remarkably, in spite of apparently normal induction of 24-OHase expression (Fig. 4), regulation of all of the target genes tested in SCC9 cells was either lost, or in the case of calmodulin and GAP SH3 binding protein, attenuated (Fig. 6C). These results provide a strong correlation between increased resistance to the antiproliferative effects of 1α,25(OH)$_2$D$_3$ and a broad but selective loss of 1α,25(OH)$_2$D$_3$ target gene regulation in the presence of apparently normal levels of functional VDR.

EB1089 Treatment Induces Expression of GADD45α Protein and Enhances Formation of GADD45α-Proliferating Cell Nuclear Antigen (PCNA) Complexes

One of the more intriguing genes identified from the array screening presented above was gadd45α (Fig. 6 and Table 1). Gadd45α is a p53 target gene induced by a variety of agents that damage DNA and arrest cell growth (33–36), and overexpression of GADD45α inhibits cell proliferation (34). Ablation of the gadd45α gene provided evidence that GADD45α functions to maintain global genomic stability (35). Peak expression of GADD45α occurs in G$_1$. DNA repair is enhanced at the G$_1$/S checkpoint, and several studies have suggested that GADD45α enhances DNA repair,

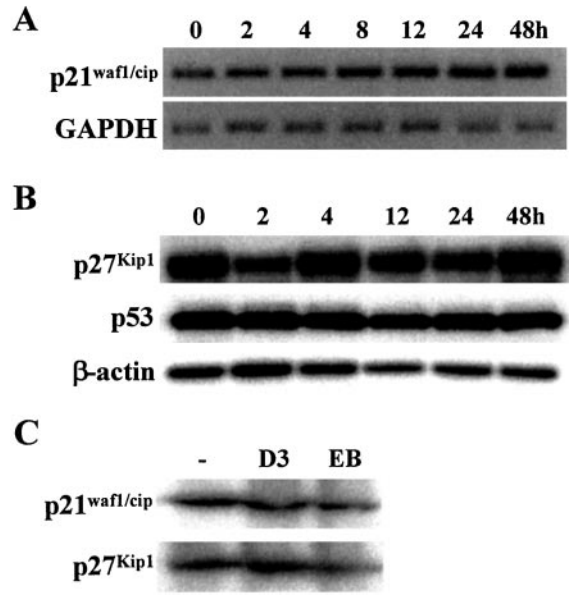

Fig. 5. EB1089-Inducible Expression of $p21^{waf1/cip1}$, but Not $p27^{kip1}$ or p53, in SCC25 Cells

A, The effect of EB1089 on expression in SCC25 cells of $p21^{waf1/cip1}$and a GAPDH control were analyzed by Northern blotting of 20 μg of total RNA from cells treated with 10 nM $1\alpha,25(OH)_2D_3$ for the times indicated. B, The effect of EB1089 on expression in SCC25 cells of $p27^{kip1}$ and p53 along with a β-actin control was analyzed by RT-PCR. Amplified products were probed with ^{32}P-labeled internal oligonucleotides as detailed in *Materials and Methods*. C, Western blotting of immunoprecipitates of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ from SCC25 cells treated for 48 h with vehicle (−), or 100 nM 1,25-$(OH)_2D_3$ (D3) or EB1089 (EB).

at least in part, through its interaction with PCNA (36–38).

Induction of gadd45α mRNA by EB1089 was only partially blocked by protein synthesis inhibitor cycloheximide (Fig. 7A, and data not shown), indicating that the effect of EB1089 is at least partially direct. In related studies, we found no effect of cycloheximide on induction of gadd45α transcripts by EB1089 in the mouse SCC line AT-84 (38a). Immunoprecipitations from control and treated SCC25 cells revealed that EB1089 induced expression of GADD45α protein (Fig. 7B), consistent with its effects on gadd45α mRNA levels. Previous studies have demonstrated that γ and UV irradiation induce GADD45α and enhance its interaction with PCNA (36, 37). It was therefore of interest to determine whether a similar interaction was induced by EB1089, which is not a DNA damaging agent. While EB1089 treatment of SCC25 cells consistently reduced expression of PCNA protein (Fig. 7B and data not shown), reciprocal coimmunoprecipitations revealed an increased association between PCNA and GADD45α in EB1089-treated cells (Fig. 7B). Thus, $1\alpha,25(OH)_2D_3$ analog EB1089 induces expression of GADD45α, leading to increased formation of GADD45α-PCNA complexes. Taken together, our results suggest that induction of GADD45α contributes to the growth-inhibitory effects of $1\alpha,25(OH)_2D_3$ and EB1089 in SCC25 cells.

DISCUSSION

The results presented above show that $1\alpha,25(OH)_2D_3$ and EB1089 were as or more potent, respectively, than 13-*cis*-RA in inhibiting growth of SCC25 cells in culture. SCC4, SCC9, and SCC15 cells were partially resistant to 13-*cis*-RA and to $1\alpha,25(OH)_2D_3$ and EB1089 (Fig. 1), raising the possibility of a common underlying mechanism of resistance. Expression of RARs β and γ is lost or reduced, respectively, in SCC4, SCC9, and SCC15 cells (14). However, no evidence was found for loss of VDR expression or function in these lines. No substantial differences were observed in induction of endogenous 24-hydroxylase gene expression, the transcription of which is controlled by a VDRE-containing promoter (21), or of a transiently transfected VDRE3-hsp68/lacZ reporter plasmid. This is consistent with other findings suggesting that VDR levels vary little among SCC lines, including SCC4 (39, 40). Our results showed that VDRs expressed in all four lines studied retained the capacity to activate transcription from VDRE-containing promoters. We have also characterized a mouse SCC line, AT-84, which is highly sensitive to $1\alpha,25(OH)_2D_3$ and EB1089 but resistant to the growth- inhibitory effects of retinoids (38a), showing that resistance to $1\alpha,25(OH)_2D_3$ and retinoids is not necessarily coupled.

Several results suggest that many factors contribute to the growth-inhibitory effects of $1\alpha,25(OH)_2D_3$ in a cell-specific manner. Transcripts encoding the cyclin-dependent kinase inhibitors $p21^{waf1/cip1}$ and $p27^{kip1}$ were strongly and rapidly up-regulated by $1\alpha,25(OH)_2D_3$ in myeloid leukemia cells, and forced expression of $p21^{waf1/cip1}$ induced myeloid cell differentiation (4, 22). Moreover, a VDRE that functioned in U937 cells was identified in the p21 promoter (4). However, the effect of $1\alpha,25(OH)_2D_3$ on $p21^{waf1/cip1}$ and $p27^{kip1}$ expression is highly cell specific. The induction of $p21^{waf1/cip1}$ mRNA by EB1089 in SCC25 cells was gradual and modest, but no effect on protein levels was observed (Fig. 5). $1\alpha,25(OH)_2D_3$ treatment modestly increased $p21^{waf1/cip1}$ protein in LNCaP prostate cancer cells (23). However, no significant effect on transcript levels and no $1\alpha,25(OH)_2D_3$-dependent induction of the $p21^{waf1/cip1}$ promoter was observed in gene transfer experiments in LNCaP cells (23). Hershberger *et al.* (6) found that $1\alpha,25(OH)_2D_3$ repressed $p21^{waf1/cip1}$ expression in the mouse SCCVII/SF line, and we have observed a similar repression of $p21^{waf1/cip1}$ transcripts and protein in the mouse SCC line AT-84 (38a).

The lack of induction of cyclin-dependent kinase inhibitors in $1\alpha,25(OH)_2D_3$- or EB1089-treated SCC25 cells led us to screen gene arrays to identify other regulated genes in SCC25 cells. A total of 38 target genes, including $p21^{waf1/cip1}$, were identified in two

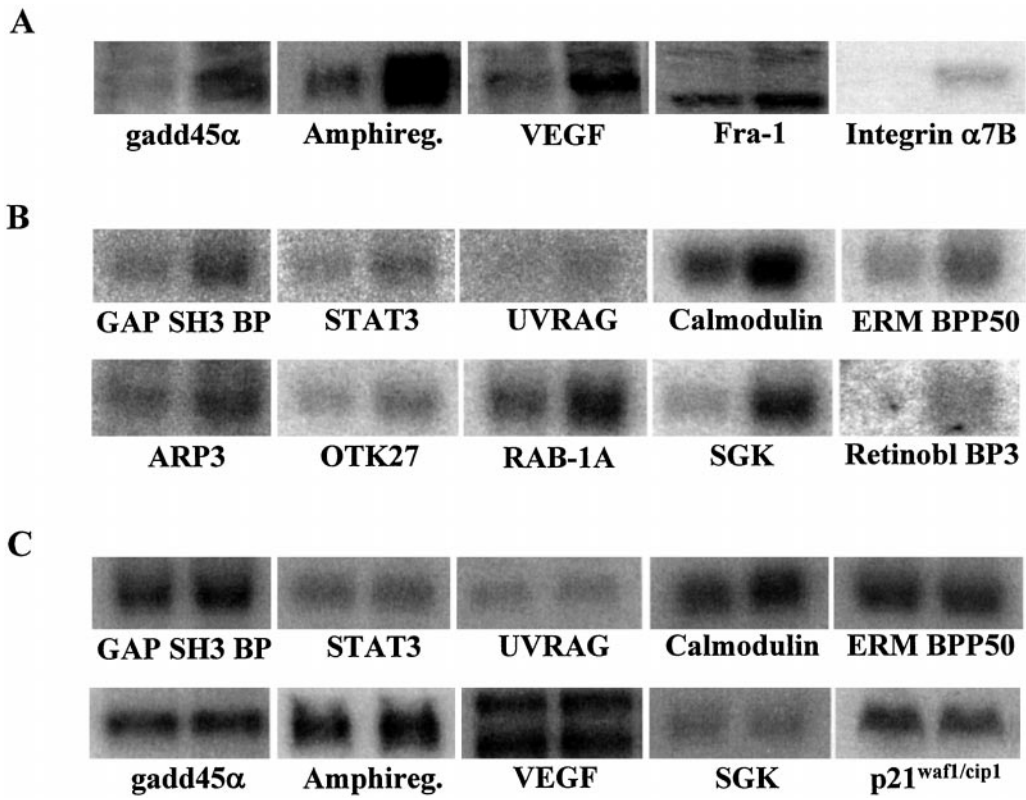

Fig. 6. Northern Analysis of EB1089-Regulated Target Gene Expression

A, Northern analyses of target genes identified using a CLONTECH Laboratories, Inc. Atlas array. Transcripts expressed in SCC25 cells encoding amphiregulin (amphireg.), GADD45α, FRA-1, integrin α7B, and VEGF are shown. Cells were treated with vehicle (*left lane*) or 10 nM EB1089 (*right lane*) for 24 h, and 1 μg of poly A+ RNA was loaded on each lane. B, Northern analyses, performed as in panel A, of target genes identified using a Research Genetics, Inc. gene filter, as follows: GAP SH3 BP, GAP SH3 binding protein; STAT3; UVRAG, UV resistance-associated gene; calmodulin; ERM BPP50, ezrin-radixin-meoisin binding phosphorprotein-50; ARP3, actin-related protein 3; OTK27; RAB-1A, ras-related protein 1A; SGK, serum- and glucocorticoid-inducible kinase; Retinobl BP3, retinoblastoma binding protein 3. C, Northern analysis of target gene regulation in SCC9 cells. Cells were treated and blots were performed as in panel A. Note that GAPDH controls were performed for blots in A–C and showed no significant variations (not shown).

screens of more than 4,500 genes (Table 1). The 32 targets identified on the Research Genetics, Inc. filter were retained after 9 sets of cross- comparisons of data derived from screening with probe derived from vehicle- and EB1089-treated SCC25 cells, using a minimum induction of 1.5-fold as a cut-off. We confirmed that 10 of 10 candidates analyzed by Northern blotting showed 1α,25(OH)$_2$D$_3$-regulated expression (Fig. 6), indicating that the data obtained from the array screening are highly reliable. Most genes were up-regulated 2- to 4-fold, a range in good agreement with that of up-regulated targets identified in a similar screen of thyroid hormone- regulated genes (33), and generally more modest than the levels of gene regulation observed by forced overexpression of the tumor suppressor genes BRCA1 (41) and WT1 (42).

The genes identified in this study encode several different classes of proteins, many of which are components of different signal transduction pathways. They include cell adhesion proteins (*e.g.* galectin-2, integrin α7B), growth factors (*e.g.* amphiregulin, VEGF), cytoskeletal proteins (*e.g.* actin-related protein 3), protein kinases (*e.g*. serum- and glucocorticoid-regulated kinase, sgk), other intracellular signaling molecules, and transcription factors (AP-4, STAT-3, FRA-1). Some of the genes identified here have been implicated in regulation of the cell cycle and growth arrest. One example is serum- and glucocorticoid-inducible kinase, SGK, which is shuttled between the nucleus and the cytoplasm during the cell cycle. Its forced retention in either compartment suppressed serum-induced growth and DNA synthesis in mammary tumor cells (43).

We also found that 1α,25(OH)$_2$D$_3$ and EB1089 induced expression of gadd45α, which like p21$^{WAF1/CIP1}$ is a p53 target gene. However, neither compound affected p53 expression in SCC25 cells. A similar induction of GADD45α expression by 1α,25(OH)$_2$D$_3$ and EB1089 was observed *in vitro* and *in vivo* in the murine SCC line AT-84 under conditions in which expression of p53 was unaffected and p21$^{WAF1/CIP1}$ was repressed. In contrast, DNA damaging agents induced p53, p21$^{WAF1/CIP1}$, and

Table 1. Summary of Target Genes Identified Using cDNA Microarrays

Function	Gene	Array	Fold Induction (± SEM)	RG cDNA id	UniGene Accession No.
Cell adhesion/Extracellular matrix	*Integrin α7B*	C	NA	NA	AF032108
	GALECTIN-2	RG	2.75 (0.77)	1472743	AA872397
	Ninjurin1	RG	2.45 (0.49)	744917	AA625806
Growth factors/receptors	*Amphiregulin*	C	NA	NA	M30704
	VEGFα	C	NA	NA	AF022375
	Macrophage stimulating 1 (hepatocyte growth factor-like)	RG	2.62 (0.72)	72395	T51539
	Platelet-derived growth factor receptor, β-polypeptide	RG	2.35 (0.58)	40643	R56211
Cytoskeletal proteins	*Ezrin-radixin-moesin binding phosphoprotein-50*	RG	3.93 (1.70)	773286	AA425299
	Actin-related protein (ARP3)	RG	3.05 (0.90)	593251	AA164562
	Sarcospan-2 (SPN2)	RG	2.78 (0.60)	1049330	AA620859
	Keratin 4	RG	1.95 (0.17)	1035889	AA629189
Signal transduction	*GADD45α*	C	NA	NA	HUMGADD45
	Calmodulin	RG	5.16 (2.50)	321389	W44860
	Ras-related protein (RAB-1A)	RG	3.95 (1.76)	293715	N69689
	GAP SH3 binding protein	RG	3.21 (1.21)	564756	AA129537
	SGK	RG	3.05 (0.90)	840776	AA486082
	Protein kinase (MLK-3)	RG	2.72 (0.91)	146868	R80779
	Retinoblastoma binding protein	RG	2.48 (0.51)	768260	AA424950
	Aplysia ras-related homolog 12	RG	2.50 (0.74)	897158	AA676955
	LDL-receptor related protein	RG	1.98 (0.28)	810551	AA64566
	Melanoma differentiation-associated gene (MDA-7)	RG	1.60 (0.25)	712049	AA281635
Transcription factors/ nuclear proteins	*Fra-1*	C	NA	NA	HSFRA1M
	OTK27	RG	3.44 (1.20)	950709	AA608583
	Activating protein 4 (AP-4)	RG	3.28 (1.04)	713839	AA284693
	STAT 3 (acute response factor)	RG	2.95 (1.02)	725746	AA399410
	IL-4 Stat	RG	2.65 (0.74)	85541	T72202
	Cytokine-inducible nuclear protein	RG	2.44 (0.55)	840683	AA488072
	GCN5-like 1	RG	1.81 (0.20)	230218	H94857
Other	SCP-1	RG	3.48 (1.76)	1031799	AA609655
	HsPex13p	RG	3.28 (1.24)	128783	R16849
	T245 protein (T245)	RG	2.92 (0.81)	252382	H87106
	IAI.3B	RG	2.86 (0.59)	882511	AA676470
	Enigma gene	RG	2.67 (0.57)	502682	AA127096
	Homo sapiens PAP	RG	2.54 (0.52)	511066	AA100296
	Prostaglandin-endoperoxide synthase 1 (PGG/H synthase and cyclooxygenase)	RG	2.52 (0.50)	811927	AA454668
	PM5 protein	RG	2.50 (0.56)	884673	AA629923
	UV Radiation resistance associated gene	RG	2.48 (0.53)	823901	AA490501

EB1089-dependent regulation of genes listed in *italics* has been verified by Northern blotting. RG, Research Genetics Gene Filter; C, Clontech Atlas Array; LDL, low-density lipoprotein; PAP, poly(A) polymerase.

GADD45α in AT-84 cells (38a). Taken together, these results suggest that $1\alpha,25(OH)_2D_3$- and EB1089-dependent induction of gadd45α occurs by a p53-independent mechanism.

Consistent with its effects on gadd45α mRNA, EB1089 treatment of SCC25 cells enhanced expression of GADD45α protein and stimulated formation of GADD45α-PCNA complexes. Previous studies have shown that DNA damaging agents, such as γ or UV irradiation, induce formation of GADD45α-PCNA complexes (36, 37). Induction of GADD45α-PCNA complexes by EB1089, which is a growth inhibitor, but not a DNA damaging agent, indicates that increased DNA damage is not necessary to induce complex formation.

PCNA function is required for DNA replication in S phase, and for DNA repair through its association with

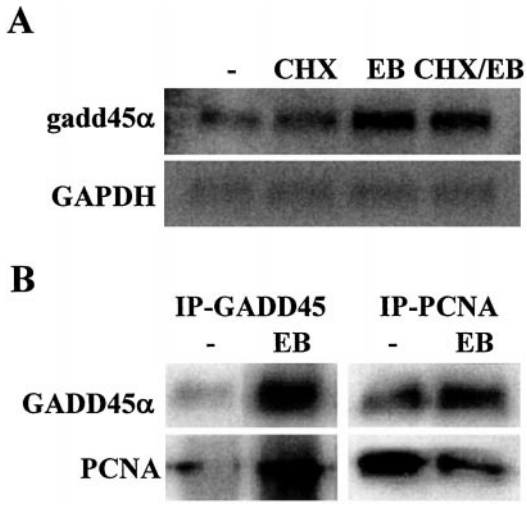

Fig. 7. Induction of gadd45α Expression and Enhanced Formation of GADD45α-PCNA Complexes in EB1089-Treated SCC25 Cells

A, The effect of 200 nM cycloheximide (CHX) on induction of gadd45α expression by 100 nM EB1089 (EB) was analyzed by Northern blotting. SCC25 cells were treated for 48 h with vehicle (−), cycloheximide, or EB1089 as indicated. B, Induction GADD45α protein and association of GADD45α with PCNA was assessed by reciprocal coimmunoprecipitation of extracts of SCC25 cells treated for 48 h with 100 nM EB1089 (EB) using either anti-GADD45α antibody (*left panel*) or anti-PCNA antibody (*right panel*) followed by Western blotting (see *Materials and Methods* for details).

polymerases δ and ε (44). Association of GADD45α with PCNA is considered to divert PCNA from sites of DNA replication to sites of DNA repair. GADD45α modifies DNA accessibility on damaged chromatin and can stimulate DNA repair *in vitro* (36, 45, 46). In addition, DNA damaging agents induce changes in the nuclear distribution of PCNA (47). It should be noted, however, that PCNA also interacts with a number of other regulatory proteins, including p21$^{WAF1/CIP1}$ (48), at sites that overlap those recognized by GADD45α (49). The relative roles and importance of interactions of p21$^{WAF1/CIP1}$ and GADD45α with PCNA remain to be fully elucidated. Nonetheless, the induction of GADD45α expression and its central role in enhancing DNA repair suggest that treatment of SCC cells with 1α,25(OH)$_2$D$_3$ or EB1089 would provide a genoprotective effect. This would be an important characteristic of a potential chemopreventive agent.

The observation that 1α,25(OH)$_2$D$_3$ induced expression of VEGF in SCC cells was surprising given that increased VEGF levels are associated with tumor vascularization and tumor progression (50). Elevated VEGF levels have been correlated with a higher rate of disease recurrence and a shorter disease-free interval in SCC of the oral cavity (51). These results highlight the complexity of cellular responses to growth regulators such as 1α,25(OH)$_2$D$_3$ and its analogs, where a combination of regulatory signals is induced under conditions in which the overall effect of 1α,25(OH)$_2$D$_3$ is growth inhibitory. It should also be noted that 1α,25(OH)$_2$D$_3$-regulated expression of VEGF is highly cell specific. We did not observe any induction of VEGF expression in MCF-7 and MBA-MD231 breast cancer or LNCaP prostate cancer cells (data not shown), whereas others have shown that VEGF expression is regulated by 1α,25(OH)$_2$D$_3$ in osteoblast-like cells (29, 30).

The partial resistance of SCC9 cells to the growth-inhibitory effects of 1α,25(OH)$_2$D$_3$ correlated with broad deregulation of target gene expression (10 of 10 genes tested). It is unlikely that loss of regulation arises through repressed expression due to target gene methylation, since transcripts of all genes refractory to 1α,25(OH)$_2$D$_3$ were detected in vehicle-treated SCC9 cells (Fig. 6). It is possible that 1α,25(OH)$_2$D$_3$-dependent induction of these genes requires synergism of the VDR with other transcription factor(s) or downstream regulators, the function of which is defective in SCC9 cells. Such factors would not be required for regulated expression of the endogenous 24-hydroxylase gene or the synthetic VDRE3-hsp68 promoter. One possible candidate is AP1, whose function is enhanced by 1α,25(OH)$_2$D$_3$ signaling (52–54). However, this enhancement apparently requires, at least in part, up-regulation of expression of AP1 components, particularly c-jun. We have also found here that 1α,25(OH)$_2$D$_3$ modestly up-regulates fra-1 mRNA levels. This suggests that if loss of induced AP1 activity contributes to deregulation of 1α,25(OH)$_2$D$_3$ target gene expression in resistant SCC lines, it may not be a primary defect. It should also be noted that we have tested the effect of cycloheximide on the six target genes identified on the Atlas array, p21$^{waf1/cip1}$, amphiregulin, VEGF, fra-1, gadd45α, and integrin α7B, and found in each case there was no effect on 1α,25(OH)$_2$D$_3$-stimulated expression (Fig. 7, and data not shown). Therefore, in these instances, the stimulatory effect of 1α,25(OH)$_2$D$_3$ did not require protein synthesis. Moreover, with the exceptions of integrin α7B and fra-1, which were not tested, regulation of all of these genes was lost in SCC9 cells (Fig. 6).

In summary, our studies have shown that 1α,25(OH)$_2$D$_3$ analogs can be potent inhibitors of SCC proliferation and control the expression of several regulators of cell proliferation. However, partial resistance to 1α,25(OH)$_2$D$_3$ can arise even in the presence of apparently normal levels of functional VDR. Resistance arises from a broad, but selective, loss in 1α,25(OH)$_2$D$_3$-regulated gene expression in the presence of normal levels of functional VDRs.

MATERIALS AND METHODS

Plasmids and Reagents

The VDRE3-hsp68-lacZ reporter contains three VDREs (20), inserted upstream of minimal hsp68 promoter in the plasmid p610AZ (55). The plasmid tk-LUC contains a truncated Her-

pes Simplex Virus thymidine kinase promoter inserted upstream of a promoterless luciferase reporter gene in pXP1 (56). 1α,25(OH)$_2$D3 and EB1089 were kindly supplied by Dr. Lise Binderup (Leo Laboratories, Ballerup, Denmark). 13-*cis*-RA was purchased from ICN Biochemicals, Inc. (Costa Mesa, CA). All hormones were dissolved in dimethylsulfoxide (DMSO), and stock solutions were stored in the dark at −20 C.

Tissue Culture

The SCC lines, SCC4, SCC9, SCC15, and SCC25, obtained from the American Type Culture Collection (ATCC, Manassas, VA), were cultured under recommended conditions. Effects of 1α,25(OH)$_2$D$_3$, EB1089, and 13-*cis*-RA on cell growth were analyzed by seeding cells in 6-well plates at 15,000 cells per well in 2 ml of culture medium containing charcoal-stripped serum. Media were changed after 24 h to charcoal-stripped medium containing vehicle or ligand as indicated. Media were changed every 48 h and fresh ligand added. Cells were harvested by washing with 2 ml of PBS and incubation with 0.7 ml of 0.25% trypsin-EDTA. Cell numbers were determined using a hemacytometer. Four grid sections were counted for each well and the results were averaged. All treatments were performed in triplicate.

Transient Transfections

SCC cells were grown to 60% confluency in six-well plates in charcoal-stripped medium, washed with 2 ml of Opti-MEM I-reduced serum media (Life Technologies, Inc., Burlington, Ontario, Canada), and cultured in 1 ml of Opti-MEM I. Cells were transfected with 500 ng of VDRE3-LacZ reporter plasmid and 500 ng of tk-LUC internal control using Lipofectin (Life Technologies, Inc.) according to the manufacturer's protocol. After 18 h media were replaced with charcoal- stripped medium containing ligands as indicated. Cells were lysed 24 h later using lysis buffer (Promega Corp., Madison, WI), and β-galactosidase assays were performed as described (57). Transfections were performed in triplicate and standardized using the Luciferase Assay System with reporter lysis buffer (Promega Corp.).

RNA Isolation and Northern Blotting

Cells were grown in 100-mm dishes. Media were replaced with charcoal-stripped medium containing ligand as indicated. Total RNA was extracted with TRIZOL (Life Technologies, Inc.). PolyA+ RNAs were isolated using an Oligotex mRNA Kit (QIAGEN, Valencia, CA). One microgram of polyA+ RNA was separated on a 1.0% agarose gel containing 6.3% formaldehyde, 20 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.0), 15 mM sodium acetate, and 1 mM EDTA. Separated RNAs were transferred to a Nylon membrane (Hybond-N+, Amersham Pharmacia Biotech, Baie d'Urfe, Quebec), which then was soaked in 3×saline-sodium citrate (SSC) and 0.1% SDS at 50 C, and prehybridized at 42 C in 50 mM phosphate buffer, pH 6.5, 50% formamide, 5× SSC, 10% Denhardt's solution containing 250 μg/ml sheared, denatured salmon sperm DNA. Hybridization was carried out in the same solution by the addition of ^{32}P-labeled cDNA probes. Membranes were washed four times in 2×SSC and 0.2% SDS for 5 min, three times in 0.1×SSC and 0.2% SDS for 30 min at 50 C, dried, and autoradiographed. All blots were performed at least three times with independent preparations of RNA.

RT-PCR

Ten micrograms of total RNA were subjected to oligo dT priming first-strand cDNA synthesis by SuperScript II (Life Technologies, Inc., Burlington, Ontario, Canada). Twenty microliter aliquots were diluted 5-fold with water. For RT-PCR analysis of p53 and p27 ^{kip1} mRNA, expression of 1 μl of RT reactions was analyzed by PCR amplification as follows: 30 sec denaturation at 94 C, 45 sec elongation at 72 C, and 30 sec annealing starting at 60 C, down 1 C per cycle to 55 C, and continuing 20 cycles of amplification (94 C for 30 sec, 57.5 C for 30 sec, 72 C for 45 sec). Complementary DNAs for p53 and p27 ^{kip1} were amplified using 5′-primer 5′-CAAGTCTGTGACTTGCACGTA-3′ and 3′-primer 5′-TTCTT-GCGGAGATTCTCTTCC-3′ for p53, and 5′-primer 5′-CCG-GAATTCATGTCAAACGTGCGAGTGTCT-3′ and 3′-primer 5′-CCGGAATTCTTACGTTTGACGTCTTCTGAGGC-3′ for p27^{kip1}. For β-actin, 1 μl of RT reaction was subjected to 18 cycles of amplification (95 C for 30 sec, 56 C for 1 min, 72 C for 25 sec) using 5′-primer 5′-GCTGTGCTATCCCTGTACGC-3′ and 3′-primer 5′-CCAATGGTGATGACCTGGC-3′. All of the above reactions were performed in 25 μl of 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-Cl (pH 9.0) using 2.5 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). PCR reactions were loaded on a 2% agarose gel, transferred for Southern blotting to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech), and fixed by UV cross-linker. The membrane was soaked in 3× SSC and 0.1% SDS at 50 C, and prehybridized at 42 C in 50 mM phosphate buffer, pH 6.5, 5× SSC, 10% Denhardt's solution containing 250 μg/ml sheared and denatured salmon sperm DNA. Hybridization was carried out in the same solution by the addition of ^{32}P end-labeled oligonucleotides 5′-CTACAAGCAGTCACAGCACAT-3′ for p53, 5′-CTAACTCTGAGGACACGCATT-3′ for p27^{kip1}, and 5′-CGAGAAGCTGTGCTACGTCG-3′ for β-actin. After hybridization, the membrane was washed four times in 2× SSC and 0.2% SDS for 5 min, three times in 0.1× SSC and 0.2% SDS for 30 min at 50 C, dried, and autoradiographed. All experiments were repeated at least three times.

Immunoprecipitation and Western Blotting

After incubation with ligands, cells were washed twice with PBS and harvested by scraping in 1 ml of PBS and centrifuged at 4 C. The pellet was resuspended in 500 μl of ice-cold lysis buffer (10 mM Tris-HCl, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% NP40) containing protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany), incubated on ice for 10 min. Lysates were centrifuged at 4 C (14,000 rpm, 10 min), and supernatants were recovered. For p21$^{WAF1/CIP1}$ and p27^{KIP1} immunoprecipitations, protein extracts (200 μg) were immunoprecipitated at 4 C overnight with 3 μg of F-5 and F-8 anti-p21$^{WAF1/CIP1}$ and −p27^{KIP1} monoclonal antibodies, respectively (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) using 30 μl of 50% slurry protein S-Sepharose (Amersham Pharmacia Biotech). Beads were centrifuged, and pellets were washed four times each with lysis buffer and boiled for 3 min in 2× SDS-polyacrylamide gel loading buffer. Immunoprecipitates were resolved on 20% SDS-polyacrylamide gels and analyzed by Western blotting with the same antibodies. Immunoprecipitations of GADD45α and PCNA were performed with anti-GADD45α antibody 4T-27 or with anti-PCNA antibody PC-10 (Santa Cruz Biotechnology, Inc.). Immunoprecipitates were harvested, processed for Western blotting as above and probed with anti-GADD45 antibody (H-165) (Santa Cruz Biotechnology, Inc.) or with anti-PCNA (PC-10) (Santa Cruz Biotechnology, Inc.).

Western analysis of VDR expression was performed with 30 μg of total cell protein resolved on a 15% SDS-polyacrylamide gel. VDRs were probed with 800 ng of a rabbit polyclonal anti-VDR antibody (Santa Cruz Biotechnology, Inc.). Proteins were detected by enhanced chemiluminescence (ECL; NEN Life Science Products, Boston, MA).

Flow Cytometry and TUNEL Assays

SCC25 cells treated with 100 nM EB1089 or DMSO for 72 h were harvested with 0.25% trypsin-EDTA, fixed with 70% ethanol for 1 h at 4 C, treated with 200 μg/ml RNase A for 30 min, stained with 5 μg/ml propidium iodide for DNA, and analyzed for cell cycle status by flow cytometry (Becton Dickinson and Co., Franklin Lakes, NJ). Experiments were repeated three times. TUNEL assays were performed using an Apoptag kit (Intergen, Purchase, NY) according to the manufacturer's instructions. Briefly, after incubation with vehicle or ligand, cells were fixed for 15 min in 1% paraformaldehyde, washed twice with PBS, and stored in 70% ethanol at −20 C. Cells (100 μl) were then incubated for 30 min at 37 C with terminal deoxynucleotidyl transferase and digoxigenin-dUTP. After two washes with 0.1% Triton X-100 in PBS, cells were incubated with fluorescein-conjugated antidigoxigenin antibody for 30 min at room temperature. After two washes with 0.1% Triton X-100 in PBS, cells were treated with RNase A and processed for flow cytometry as above.

Array Screening

SCC25 cells were treated for 24 h with DMSO or EB1089 (100 nM). Atlas cDNA Expression Arrays containing 588 genes (CLONTECH Laboratories, Inc. Palo Alto, CA) were screened with 100 ng of polyA+ RNA. GF211 Named Human Genes arrays containing more than 4,000 genes (Research Genetics, Inc.) were probed with 1 μg of total RNA. Probe preparation and array screening were carried out according to manufacturers' instructions. Duplicate Atlas arrays were screened twice each with probe derived from control or treated cells and arrays were visualized by autoradiography. Genes that appeared reproducibly regulated were studied by Northern analysis. GF211 filters were probed three times each with probe derived from control cells and EB1089-treated cells, and visualized by phosphorimaging. Relative expression levels were compared using Pathways software (Research Genetics, Inc.). Genes that were up-regulated at least 1.5-fold in nine sets of cross-comparisons were retained. Of these, 10 were further analyzed by Northern analysis using cDNA probes from Research Genetics, Inc.

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