The E1A Oncogene Induces Resistance to the Effects of 1,25-Dihydroxyvitamin D₃ on Inhibition of Growth of Mouse Keratinocytes

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Abstract

1,25-Dihydroxyvitamin D₃ [1,25-(OH)₂D₃] inhibited DNA synthesis in transformed mouse keratinocytes (Pam212) in a time- and dose-dependent manner as measured by [³H]thymidine incorporation. To investigate the mechanism through which 1,25-(OH)₂D₃ acts, we examined its effects on Pam212 cells further transformed with the E1A oncogene. Here, we show that transformation of the cells with the E1A oncogene induced resistance to the effects of 1,25-(OH)₂D₃ on inhibition of growth of Pam212 cells. While 1,25-(OH)₂D₃ treatment increased the level of expression of vitamin D receptor mRNA 20-fold in parental cells, the E1A-transformed cells failed to express vitamin D receptor mRNA even after treatment with 1,25-(OH)₂D₃. Transfection of the E1A-transformed cell line with an expression construct encoding the vitamin D receptor restored receptor expression as well as the inhibition of growth by 1,25-(OH)₂D₃. These results suggest that one of the mechanisms for acquisition of 1,25-(OH)₂D₃ resistance induced by E1A may involve loss of vitamin D receptor inducibility by 1,25-(OH)₂D₃.

Introduction

Although 1,25-(OH)₂D₃ is a major regulator of calcium homeostasis (1), it has also been shown to differentiate leukemia cells in vitro (2—4), to inhibit growth of cancer cells (5), and to inhibit chemical carcinogenesis (6, 7). Since vitamin D₃ and its synthetic analogues are potent candidate compounds for the treatment of patients with cancer and skin diseases, like psoriasis, understanding of their mechanisms of action is of importance (8, 9).

The biologically active form of vitamin D, 1,25-(OH)₂D₃, mediates its target organ response through a nuclear-associated receptor protein (10). Regulation of the level of VDR is an important mechanism that modulates cellular responsiveness to 1,25-(OH)₂D₃ (10, 11). In a variety of cells, regulation of VDR expression has been shown to be closely associated with the regulation of cellular proliferation (12). A key regulator of vitamin D receptor gene expression has been shown in several cell lines (13—17). However, the molecular mechanisms regulating expression of the VDR gene are not yet known.

In Pam212 keratinocytes (a transformed cell line derived from a mouse squamous cell carcinoma), the product of the adenovirus early region 1A (E1A) has been found to induce total resistance to inhibition of growth by TGF-β (18), dexamethasone (19), and cyclic AMP (20). This effect depends on the ability of E1A to bind to at least four different cellular proteins (18). In the present study, we have examined whether E1A can also interfere with the growth inhibition of these cells by 1,25-(OH)₂D₃. We show that E1A induces resistance to the inhibition of growth by 1,25-(OH)₂D₃ by blocking autoregulated VDR gene expression and that expression of recombinant VDR restores the inhibitory action of 1,25-(OH)₂D₃ on growth.

Materials and Methods

Reagents. 1,25-(OH)₂D₃ and all-trans-retinoic acid (Hoffmann-LaRoche, Nutley, NJ) were dissolved in ethanol to 1 mM and kept at −20°C under nitrogen gas to prevent oxidation. Dilutions were made in ethanol and then used at 1:1000 in assay media. Stock solutions of TGF-β₁ (R & D Systems, Minneapolis, MN) were stored at −70°C in 4 mM HCl-1 mg/ml bovine serum albumin. The carrier controls consisted of 1:1000 dilutions of either ethanol or 4 mM HCl-1 mg/ml bovine serum albumin. Pam212 cells stably transfected with E1A were described previously (18).

Cell Culture. The transformed cells derived from the mouse keratinocyte cell line Pam212 were maintained in growth media [Dulbecco’s modified essential medium, 10% calf serum, antimycotic, and G418 (all from Gibco-BRL)]. For the assays, the cells were trypsinized and washed once with growth media and once with assay media (growth media with only 0.2% calf serum). For growth inhibition assays, the cells were plated in 24-well dishes at 2.5 × 10⁴ cells/500 μl/well. For RNA extraction, the cells were plated at 2.5 × 10⁵/50 ml/500-cm² cell tray. In either assay the cells were allowed to adhere overnight before the test substances were added.

Growth Inhibition Assay. The assay has been described previously (21). In brief, the test substances and carriers were added in 500 μl of fresh assay media and then incubated for the appropriate times. The cells were pulse-labeled for 2 h with 0.5 μCi of tritiated thymidine (Amersham) in 50 μl assay media. The cells were fixed for 1 h in a solution of 75% methanol and 25% acetic acid at room temperature, washed twice with 2 ml of 80% methanol, incubated at 37°C with 500 μl of trypsin for 30 minutes, fully solubilized with 500 μl of 1% sodium dodecyl sulfate for 5 min, and finally transferred to scintillation vials for counting.

RNA Extraction and Northern Blots. Total RNA was extracted from the cells. Three days after addition of the test substances, the cells were rinsed twice with 10 ml of PBS and then scraped from the dishes with 6 ml of 4 M guanidine thiocyanate buffer, sheared twice with a 19-gauge needle, and kept on ice. The guanidine solution was then centrifuged at 10,000 rpm for 10 min at 4°C, layered on a 5.7 M cesium chloride cushion, and spun at 30,000 rpm for 20 h at 20°C. The following day the pellets were suspended in 300 μl of diethyl pyrocarbonate-treated water, extracted once with chloroform:isoamyl alcohol (4:1), and precipitated with 300 μl of 4 M sodium acetate, pH 5. The samples were electrophoresed in a 2 M formaldehyde gel, transferred to Nylon, and hybridized. Blots were hybridized with cDNA probes for VDR (22) and glyceraldehyde-3-phosphate dehydrogenase.

Expression and Plasmid Transfection. A VDR expression plasmid (CMX-hVDR) described previously (23) was cotransfected with pCEP4 (Invitrogen), a hygromycin expression plasmid, by a calcium phosphate coprecipitation method into d1799N cells (Pam212 cells stably transfected with E1A). Forty-eight h after transfection, cells were treated with hygromycin (Calbiochem) (50 ng/ml). Hygromycin-resistant clones were isolated by limiting dilution and were individually expanded. These clones were screened for expression of VDR.
Results and Discussion

The growth of control SV2neo-transfected cells is inhibited by 1,25-(OH)₂D₃ in both a time- and dose-dependent manner (Figs. 1 and 2). Although the cells do not show a decrease in growth with less than 2 days of treatment, at even the lowest dose of 0.1 nM, they are strongly inhibited following 2 days or more of treatment with 1,25-(OH)₂D₃. In contrast, Pam212 cells transformed with the E1A cDNA (dl799N) are resistant to inhibition of growth by 1,25-(OH)₂D₃ (Fig. 2).

Treatment of the SV2neo cells with 1,25-(OH)₂D₃ markedly increased the expression of VDR mRNA (Fig. 3). Normalization of the level of the VDR mRNA with that of glyceraldehyde-3-phosphate dehydrogenase showed a 10-fold induction. All-trans-retinoic acid slightly increased the level of VDR mRNA, and the combination of 1,25-(OH)₂D₃ and all-trans-retinoic acid increased the RNA levels 20-fold over carrier control (Fig. 3). TGF-β₁ had no effect on the expression of VDR mRNA. Cells harboring an E1A gene with an intact transforming region (dl799N) showed little or no induction of VDR mRNA level after 1,25-(OH)₂D₃ treatment. These results suggest that resistance to the growth-inhibitory effects of 1,25-(OH)₂D₃ induced by the E1A protein is, in part, due to the loss of inducibility of VDR mRNA level by 1,25-(OH)₂D₃.

Since Pam212 cells expressing E1A (dl799N) had low to undetectable levels of the VDR mRNA (Fig. 3), we next studied the effects of transfecting these cells with the gene for VDR. After transfection with VDR cDNA (pCMX-VDR), hygromycin-resistant clones were expanded and examined for expression of the VDR gene. Clones dl799N-VDR1 and dl799N-VDR2 (Fig. 4, Lane 3) expressed high levels of VDR message. VDR mRNA expression was not detected in any of the three hygromycin-resistant clones which were transfected with the pCEP4 vector only (Fig. 4, Lane 1, and data not shown). Hygromycin-resistant cells that were transfected with the control pCEP4 plasmid showed little inhibition by 1,25-(OH)₂D₃, even at 100 nM.
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