1,25-Dihydroxyvitamin D Down-regulates Cell Membrane Growth- and Nuclear Growth-promoting Signals by the Epidermal Growth Factor Receptor*

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1,25(OH)2D3 antiproliferative properties are widely known. However, the molecular bases of these properties are only partially elucidated. Since 1,25(OH)2D3 effectively arrests growth in many tumors and hyperplastic tissues whose growth is driven by co-expression of EGFR and its ligand TGF-α, it was hypothesized that 1,25(OH)2D3 could affect the TGF-α/EGFR-autocrine growth loop. This study examined 1,25(OH)2D3 regulation of EGFR-growth signals, using human epidermoid A431 cells, in which the overexpression of EGFR and TGF-α constitute the major autocrine mitogenic signal. 1,25(OH)2D3 inhibited autocrine and EGFR-induced A431 cell proliferation. Furthermore, 1,25(OH)2D3 changed the cellular localization of both TGF-α and EGFR and inhibited ligand-dependent phosphorylation of EGFR and ERK1/2. In addition, 1,25(OH)2D3 impaired autocrine and EGFR-induced nuclear translocation of activated EGFR and, consequently, its binding to AT-rich DNA sequences and transcriptional activation of the cyclin D1 promoter. These results demonstrate that 1,25(OH)2D3 alters EGFR membrane trafficking and down-regulates EGFR growth signaling.

The epidermal growth factor receptor (EGFR)† is a 170-kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity (1, 2). The receptor kinase is activated by binding with at least five distinct ligands, including EGF and Transforming growth factor-α (TGF-α). Upon ligand binding, the EGFR undergoes autophosphorylation and initiates multiple intracellular signaling cascades, leading to induction of cell growth (3, 4). The best-characterized EGFR mitogenic signal is the Ras-Raf-MAPK pathway (5, 6). EGFR activation also induces other signaling pathways that turn off EGFR signaling through endocytosis. Attenuation of the signaling is important for the control of EGFR mitogenic properties (7, 8).

In addition to its mitogenic signaling, which is common to other tyrosine kinase transmembrane receptors, the EGFR promotes growth by binding to AT-rich DNA sequences (ATTR sequences), thus acting as a transcription factor or a co-activator for cyclin D1 gene expression in cells overexpressing the EGFR (9). This novel EGFR function provides an explanation for the strong correlation between nuclear EGFR localization and highly proliferating activity that has been observed in numerous tissues (10–13).

TGF-α is an important growth factor in normal physiology as well as in hyperproliferative disorders and oncogenesis (14, 15). The soluble form of TGF-α is produced from the proteolytic cleavage of a transmembrane TGF-α precursor (16, 17). Both TGF-α isoforms bind EGFR and activate signal-transduction pathways that enhance cell proliferation (18, 19). Elevated TGF-α has been implicated not only in the genesis and maintenance of tumors, but also in non-neoplastic hyperproliferative disorders such as psoriasis (20) and the hyperparathyroidism induced by renal failure (21). Secretion of TGF-α in particular is potent mitogenic because its dissociation from EGFR after endocytosis promotes receptor recycling, rather than degradation, and allows unabated signaling in the presence of a continuous ligand source (22).

In addition to its well-studied role in calcium metabolism, the hormonal form of vitamin D (1,25(OH)2D3) is known to suppress normal and malignant cell growth (23, 24). 1,25(OH)2D3 therapy is highly effective in arresting keratinocyte growth and the progression of psoriatic lesions (25, 26) as well as in preventing TGF-α-induced parathyroid hyperplasia (21, 27). 1,25(OH)2D3 antiproliferative actions require the vitamin D receptor (VDR) (24, 28–31), a member of the steroid-receptor superfamily. Several mechanisms have been implicated in 1,25(OH)2D3 arrest of the cell cycle at the G0/G1 transition (32) including reduction of c-myc expression (33) or activation of the cyclin-dependent kinase inhibitor p21 (34).

In human epidermoid A431 cells, like in many aggressively growing tumors, enhanced co-expression of TGF-α and EGFR generates an autocrine growth loop that constitutes the major mitogenic signal (35, 36). This result is also supported by the fact that the use of specific inhibitors of the EGFR tyrosine
kinase significantly arrests growth in A431 cells and in TGF-α-driven tumors (37, 38).

The effectiveness of 1,25(OH)2D3 in suppressing TGF-α-induced growth in psoriasis and renal hyperparathyroidism led us to hypothesize that 1,25(OH)2D3 could be affecting the TGF-α/EGFR-autocrine growth loop. To test this hypothesis, we used the human epidermocarcinoma cell line A431.

In this study, we present evidence for a novel mechanism for the antiproliferative properties of 1,25(OH)2D3. 1,25(OH)2D3 alters EGFR membrane trafficking, down-regulates EGFR-growth signaling, and impairs binding of nuclear EGFR to cognate DNA sequences, which in turn decreases its transcriptional activity on the cyclin D1 promoter.

EXPERIMENTAL PROCEDURES

Cell Culture—A431 (ATCC, Manassas, VA), NR6, or HeLa cells were cultured in Dulbecco’s modified Eagle medium (Invitrogen) containing 4 mM l-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate supplemented with 10% fetal bovine serum (Fisher) and incubated at 37 °C in a 5% CO2 atmosphere. For 1,25(OH)2D3 treatment, cells were cultured in 6-well plates at a concentration of 5 × 105 cells/well in Dulbecco’s modified Eagle medium supplemented with 2% fetal bovine serum either in the presence or absence of 100 nM 1,25(OH)2D3 for 24 h (a gift from Dr. Uskokovic, Hoffman-La Roche). After this incubation, the medium was changed to serum-free Dulbecco’s modified Eagle medium containing 1% bovine serum albumin (Aldrich) in the presence or absence of 100 nM 1,25(OH)2D3, and cell growth was measured after 24 h. EGF (Calbiochem) treatment was performed at the concentration and time of exposure specified in the respective protocols.

Analysis of DNA Synthesis—After the specified treatment, cell growth was measured by thymidine incorporation into DNA. Cells were incubated with 1 μCi/ml of methyl-[^3]H]thymidine (Amersham Biosciences) for 4 h and washed three times with phosphate-buffered saline. Cold 10% (v/v) trichloroacetic acid was then added, and cells were solubilized with 1 M NaOH. Tritium was measured by scintillation counting (Taurus, Micromedic).

EGF Binding Assay—Mouse EGF (Calbiochem) was iodinated with 125I (PerkinElmer Life Sciences) using IODO-BEAD (Pierce) according to the manufacturer’s protocol. EGF binding was measured as previously described (39). Briefly, after treatment, cells were washed with binding buffer (α-modified Eagle’s medium, 13 mM HEPES, pH 7.4, with 0.3% dialyzed fetal bovine serum, 2 mM l-glutamine, 1 mg/ml bovine serum albumin, and 50 mg/l penicillin/streptomycin) at 37 °C, and allowed to bind 100 pM [125I]-EGF at 4 °C for 2 h. Cells were then washed five times with cold binding buffer to remove the unbound 125I-EGF. Cells were lysed overnight at 4 °C in 1 N NaOH, and surface-bound 125I-EGF was measured with a gamma counter (Apex, Micromedic). Non-specific binding (<4%) was assessed by co-incubation of radiolabeled EGF with 200 mM radioident human EGF (Aldrich).

EGFR and ERK1/2 Phosphorylation Assay—Control and 100 nM 1,25(OH)2D3-treated cells were incubated with binding buffer containing no EGF or 17 nM EGF for 20 min at 37 °C. At the end of the pulse, cells were washed and lysed in ice-cold lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 10 mM NaF, and a protease inhibitor mixture (Roche Molecular Biochemicals) pH 7.2). The lysate was vortexed and clarified by centrifugation at 16,000 × g for 15 min at 4 °C. Protein concentration in cell lysates was determined using a detergent-compatible protein assay (Bio-Rad). 100 μg of total protein from cell lysates was immunoprecipitated overnight at 4 °C with anti-EGFR antibody (Ab-5 monoclonal antibody (Oncogene Research Products) or Ab-13 monoclonal antibody (NeoMarkers)) bound to Protein A-Sepharose (Amersham Biosciences). Immunoprecipitates were analyzed by 7.5% SDS-PAGE. For ERK1/2 activation analysis, 30 μg of total protein from lysates were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane using a wet transfer apparatus (Bio-Rad). Membranes were probed with antibodies against total EGFR (Ab-5 monoclonal antibody (Oncogene Research Products) or Ab-13 monoclonal antibody (NeoMarkers)) bound to Protein A-Sepharose (Amersham Biosciences). Immunoblots were developed using supersignal reagents (Pierce). Gels were scanned, and the densitometric analysis was performed using Gel Pro Analyzer (Media Cybernetics, Silver Spring, MD).

Preparation of Nuclear Extracts—For preparation of nuclear extracts, we used the Nuclear Extract kit (Active Motif) according to the manufacturer’s instructions.
surface-bound 125I-EGF was measured and expressed as cpm/mg of phospho-
tyrosine-phosphorylated EGFR (pY-EGFR). For ERK1/2 phosphorylation analysis, 30 min after 1,25(OH)2D3 treatment, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and immunostained with the appropriate antibodies. Alexa Fluor 488 and 594 (Molecular Probes) were used as secondary antibodies. Slides were examined by confocal microscopy (MRC1024; Bio-Rad) using a 63× numerical aperture bright-field objective and fluorescein filter sets.

Immunohistochemistry—Control and treated cells were fixed in methanol for 10 min, quenched with 50 mM NH4Cl in phosphate-buffered saline for 10 min, and permeabilized with 0.1% Triton X-100. After 45 min blocking at room temperature, cells were exposed to a 1:250 dilution of either anti-total ERK1/2 or anti-phosphorylated ERK1/2 (New England Biolabs) at 4 °C overnight. A biotinylated anti-rabbit secondary antibody (Zymed) was applied, and immune complexes were visualized with aminoethyl carbazole (AEC) substrate chromogen. For each experiment, one hundred cells per field were counted using a high power-field objective. Four different fields were counted per well, and two wells were counted per experiment. Three independent experiments were performed. Nuclei staining positive for phosphorylated ERK1/2 were expressed as percent of positive nuclei per total number of cells counted.

Electrophoretic Mobility-Shift Assays (EMSAs)—Nuclear extracts from control and 1,25(OH)2D3-treated cells were obtained and used for EMSAs. The assays were conducted as described by Rotanides and Reich (40), using the EGFR consensus binding site (wild type: CTGGTTACCGTGTCCT) and its mutant (mutant type: CTACTCCAAGTCCACCATGACGGCTGGTTCCT) as probes, and 10 ng of total nuclear protein. For binding interference, nuclear extracts were incubated with anti-EGFR antibody (Ab-12 NeoMarkers). Bands in the dried gels were visualized by scanning densitometry after an overnight exposure, using a PhosphorImager (Amersham Biosciences).

RESULTS
1,25(OH)2D3 Inhibits Autocrine and EGF-induced Cell Growth in A431 Cells—To determine whether 1,25(OH)2D3 affects EGFR growth signal, we studied the effects of 1,25(OH)2D3 on the proliferation of A431 cells. 1,25(OH)2D3 inhibited basal-cell proliferation in a time-dependent manner, as shown by a decrease in cell number (Fig. 1A) and by reduction in thymidine incorporation into DNA and was also capable of abolishing all of the growth-stimulatory effect of EGF treatment (Fig. 1B). Lower doses of 1,25(OH)2D3 had no effect on basal proliferation rates (data not shown). This result suggests that growth inhibition by 1,25(OH)2D3 is related to its ability to block the EGFR-induced growth signal.
1,25(OH)D₃ Down-regulation of EGFR Signaling

1,25(OH)D₃ Reduces EGFR in the Plasma Membrane and Inhibits EGFR and ERK1/2 Activation—Because ligand-induced internalization of EGFR is an important mechanism to attenuate the receptor signaling (7, 8), we examined the effect of 1,25(OH)D₃ on EGFR localization and signaling. Treatment with 1,25(OH)D₃ caused a time-dependent decrease in plasma membrane-associated EGFR as shown by a significant increase of EGFR in the cytosol of treated cells (Fig. 2A) and confirmed by a 20% and 40% reduction in specific surface-bound ¹²⁵I-EGF after 24- and 48-h treatments, respectively (Fig. 2B). The effects of 1,25(OH)D₃ on EGFR localization correlated with a significant inhibition of ligand-dependent EGFR and ERK1/2 phosphorylation in 1,25(OH)D₃-treated cells (Fig. 2C) (pY-EGFR % reduction: 24 h, 65 ± 5 and 48 h, 78 ± 8; p-ERK1/2% reduction: 24 h, 45 ± 3 and 48 h, 80 ± 7). Furthermore, 1,25(OH)D₃ treatment reduced EGFR-induced tyrosine phosphorylation of the EGFR to levels even lower than those present without EGFR treatment. Shorter exposure time (20 min or 6 h) to 1,25(OH)D₃ did not affect either the localization (Fig. 3A) or the activation (Fig. 3B) of the EGFR, suggesting a VDR-dependent mechanism. These results suggest that 1,25(OH)D₃ affects EGFR localization and signaling.

1,25(OH)D₃ Inhibits Cell Proliferation and EGFR Activation in EGFR-overexpressing Cells—We next examined whether 1,25(OH)D₃ inhibition of cell proliferation and EGFR signaling was specific for A431 cells. We used NR6 mouse fibroblasts (Fig. 4, A and B), a non-tumorigenic cell line overexpressing the human EGFR (39, 45) and the human cell line HeLa (Fig. 4, C and D) expressing physiological levels of EGFR. 1,25(OH)D₃ significantly affected EGFR-dependent cell proliferation (compare Fig. 4, A and C) and EGFR phosphorylation (compare Fig. 4, B and D) in the cell line overexpressing EGFR, NR6, but not in HeLa cells. The results suggest that the effect of 1,25(OH)D₃ on EGFR-induced growth and signaling is not exclusive for A431 cells but requires EGFR overexpression.

Endogenous TGF-α Accompanies 1,25(OH)D₃-induced Change of EGFR Localization—As shown in figure 2A, 1,25(OH)D₃ reduced plasma membrane EGFR in the absence of exogenous ligand. To directly address whether the effect of the sterol on EGFR localization was related to that of the endogenous ligand, we examined TGF-α localization. As expected, in control cells, TGF-α co-localized with the EGFR to the plasma membrane (Fig. 5, A–C). After 48 h of treatment with 100 nm 1,25(OH)D₃, TGF-α localization was also changed from the plasma membrane to cytosolic vesicles and significantly co-localized with the EGFR (Fig. 5, D–F). Similar TGF-α/EGFR co-localization was found after 24 h of treatment with 1,25(OH)D₃ (data not shown). These data indicate that 1,25(OH)D₃ induced changes on both EGFR and TGF-α localization.

1,25(OH)D₃ Changes EGFR Localization from the Plasma Membrane to the Early Endocytic Compartment—We next examined the intracellular localization of the EGFR in 1,25(OH)D₃-treated cells. Co-localization studies using the early endosome autoantigen, EEA1, demonstrated that after a 48-h treatment with 1,25(OH)D₃, the EGFR predominantly localized in the early endocytic compartment (Fig. 5, G–I). There was no significant co-localization of the EGFR with the lysosomal marker Lamp1 (Fig. 5, J–L). The pattern of localization for EEA1 and Lamp1 was similar for control and 1,25(OH)D₃-treated cells (data not shown). These results indicate that 1,25(OH)D₃ changes EGFR localization from the plasma membrane to early endocytic compartments. The observation that in 1,25(OH)D₃-treated cells, EGFR did not localize in the lysosomal compartment is consistent with the lack of effects of the sterol in the total EGFR content, as shown in Fig. 2C.

1,25(OH)D₃ Prevents TGF-α/EGFR-induced Activation and Nuclear Translocation of ERK1/2—In many tumors and tumor cell lines, including A431 cells, co-expression of EGFR and TGF-α triggers an “autocrine” growth loop (35). Measured by
1,25(OH)2D3 reduces TGF-β from whole-cell lysate (Fig. 2), these results suggest that C

Alexa Fluor were used as secondary antibodies. Anti-rabbit or anti-mouse IgG conjugated with

lysosomal marker (G–I) or mouse anti-Lamp1, a

anti-EEA1, an early endosomal marker (J–L). Anti-rabbit or anti-mouse IgG conjugated with

Alexa Fluor were used as secondary antibodies.

proliferative indices, the magnitude of the autocrine growth signal directly correlates with nuclear translocation of phosphorylated ERK1/2 (42). Thus, prevention of nuclear translocation of activated ERK1/2 is an indicator of the therapeutic efficacy in counteracting TGF-β/cytic marker EEA1 in 1,25(OH)2D3-treated cells. Further confirm the effects of 1,25(OH)2D3 on TGF-β/efficacy in counteracting TGF-β/cation of activated ERK1/2 is an indicator of the therapeutic

correlation with nuclear translocation of phosphorylated ERK1/2. Thus, prevention of nuclear translocation of ERK1/2.

1,25(OH)2D3 Impairs EGFR-DNA Binding and Transactivation of the Cyclin D1 Promoter— It has been recently shown that activated EGFR translocates to the nucleus of EGFR overexpressing cells and binds to specific AT-rich DNA sequences, either directly or as a co-activator molecule, to activate cyclin D1 gene transcription (9). Given the importance of EGFR transcriptional activity in EGFR-overexpressing cells (9), we examined whether 1,25(OH)2D3 affected this novel EGFR biological function.

Using nuclear extracts from A431 cells and the described ATR sequence as a probe, the expected EGFR-DNA complex was observed (Fig. 7, A and C) (9). The specificity of EGFR-DNA binding was supported by competition of the protein-DNA complex by an excess of radioinert oligonucleotide containing wild-type ATR sequences (Fig. 7A, lanes 2 and 5 and Fig. 7C, lane 6), but not by an excess of mutated ATR sequences (Fig. 7A, lane 3 and Fig. 7C, lane 7). EGFR-DNA binding was significantly increased by EGF treatment (compare lanes 2 and 3 in Fig. 7C) and abolished by incubation with anti-EGFR antibody (Fig. 7C, lane 5). These results suggest that EGFR is a component of this protein-DNA complex.

1,25(OH)2D3 treatment for 48 h decreased EGFR binding to DNA in both EGF-treated (compare lanes 3 and 4 in Fig. 7C) and EGF-untreated cells (compare lanes 2 and 3 in Fig. 7A). This reduction in EGFR binding to ATR sequences was consistent with a significant decrease in both total and phosphorylated nuclear EGFR in 1,25(OH)2D3-treated cells, in the presence (Fig. 7D) or absence (Fig. 7B) of EGF (pY-EGFR % reduction: 48 h, 55 ± 8 and 48 h + EGF, 45 ± 4).

To test whether the effects of 1,25(OH)2D3 on EGFR-DNA binding resulted in an effect on its transcriptional activity, A431 cells were transfected with a wild-type luciferase reporter construct driven by the proximal region of the cyclin D1 promoter that contains ATR sequences (−163 wild type) or a mutant construct lacking the mentioned sequence (−163 mutant type) (9). As expected, EGF induced transcription of the wild-type, but not the mutant, construct (Fig. 7E). 1,25(OH)2D3...
control with 1,25D in Fig. 7E). These effects were exclusive for the wild-type construct.

Taken together, these data indicate that 1,25(OH)2D3 inhibits EGFR binding to its cognate DNA sequence and its transcriptional activity on the cyclin D1 promoter in both the autocrine and the EGF-activated system.

**DISCUSSION**

The present work shows that 1,25(OH)2D3 inhibits autocrine (TGF-α) and EGF-induced A431 cell proliferation, changes the localization of TGF-α and EGFR, and inhibits ligand-dependent EGFR and ERK1/2 activation. In addition, 1,25(OH)2D3 impairs autocrine and EGF-induced EGFR binding to AT-rich DNA sequences and consequently its transcriptional activation of the cyclin D1 promoter.

The use of A431 cells was important because they overexpress EGFR and its ligand TGF-α and proliferation in these cells is mainly driven by this growth signal (37, 43, 44). This result suggests that 1,25(OH)2D3 acts through the TGF-α/EGFR signaling pathway to inhibit growth. Although other anti proliferative actions of 1,25(OH)2D3 such as reduction of c-myc expression (33) or activation of the cyclin-dependent kinase inhibitor p21 (34) could contribute to the effects of the sterol in A431 cell growth, those actions are unrelated to TGF-α/EGFR signaling. Studies in normal NR6 mouse fibroblasts that overexpress the human EGFR (45, 39), and in the human cell line, HeLa, expressing physiological levels of EGFR, suggest that 1,25(OH)2D3 down-regulation of EGFR-induced growth and signaling requires EGFR overexpression and is independent of tumorigenicity (Fig. 4). This finding renders our work of potential importance in explaining the mechanisms for 1,25(OH)2D3 antiproliferative actions in many tumors and hyperplastic tissues whose growth is driven by co-expression of EGFR and its ligand TGF-α (25, 26).

At present, the gene/s and detailed molecular mechanisms mediating 1,25(OH)2D3 effects on EGFR localization and signaling are unknown. Most (if not all) 1,25(OH)2D3 biological actions are mediated by its receptor (VDR) (31). A431 cells express normal levels of VDR (46). Because the non-genomic, VDR-independent actions of 1,25(OH)2D3 occur within seconds (31), the long time of exposure required to see the effects of the sterol on EGFR signals (Fig. 3) suggests that these effects are VDR-dependent. The alteration of normal TGF-α/EGFR trafficking caused by 1,25(OH)2D3 could be an important determinant for the effects of the sterol in A431 cells. The effect of 1,25(OH)2D3 on EGFR localization seems to be specific as suggested by the lack of effect on the transferrin receptor (data not shown). Additional work will be required to fully identify the mechanisms by which 1,25(OH)2D3 induces apparent stasis of EGFR in the early endocytic compartment and affects EGFR signaling.

In addition to the potent inhibition of EGFR signaling at the plasma membrane, 1,25(OH)2D3 inhibits EGFR functions as a transcription factor (co-activator) of the cyclin D1 promoter in A431 cells (9) (Fig. 7). Several mechanisms could mediate this effect of the sterol, including either a direct effect of the 1,25(OH)2D3/VDR complex on EGFR DNA binding and transcriptional activity or inhibition of EGFR nuclear translocation by 1,25(OH)2D3. The former possibility is unlikely due to the long time required for the effect of the sterol. The latter is a likely mechanism for the effect of 1,25(OH)2D3 on EGFR transcriptional activity and is supported by the reduced amounts of nuclear EGFR in A431 cells after a 48-h treatment (Fig. 7, B and D). The prolonged treatment required to see this effect suggests a potential role of 1,25(OH)2D3/VDR in regulating proteins involved in EGFR trafficking to the nucleus. This novel aspect of the intracellular trafficking of the EGFR has abolished the EGF-induced luciferase activity (compare control + EGF with 1,25D + EGF in Fig. 7E) and also inhibited the reporter activity regulated by autocrine signaling (compare...
be recently postulated (9), and very little is known about how this process occurs. Further experimentation is needed to assess this mechanism in detail. The fact that the mutant construct, lacking ATR sequences, does not respond to 1,25(OH)2D3 rules out the possible role of VDR as a direct transrepressor of the cyclin D1 promoter.

In summary, this work presents evidence for a novel mechanism for the poorly understood antiproliferative actions of 1,25(OH)2D3 in TGF-α/EGFR-overexpressing cells.

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