Recent studies from several laboratories have shown perturbations of 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] metabolism in hypertension. While these perturbations may exert their effect on blood pressure via their actions on calcium metabolism, it is possible that this vitamin D metabolite may have direct effects on vascular smooth muscle cell (VSMC) physiology. To examine this, we studied the effect of 1,25(OH)2D3 on VSMC growth and found that this substance suppressed VSMC [3H]thymidine uptake; furthermore, this vitamin D metabolite also suppressed the stimulatory effect of epidermal growth factor (EGF) on VSMC proliferation. The concomitant presence of this substance appeared to be required for its action on VSMC growth since cells pretreated with the vitamin D metabolite for up to 72 hours and then washed of the substance grew normally and responded to EGF. Studies were also done to determine if 1,25(OH)2D3 had any effect on the function of EGF receptors on VSMC. Experiments using Iodine-125-labeled EGF showed no differences in the binding of this ligand to VSMC, either untreated or treated with 1,25(OH)2D3, which indicates the effect of the vitamin D metabolite on VSMC growth (when exposed to EGF) was not mediated by an alteration of EGF receptor function. The results of these studies have implications for the pathogenesis of vascular diseases such as hypertension and atherosclerosis. (Hypertension 1989;13:954–959)

**Materials and Methods**

**Reagents**

EGF from murine submaxillary glands, receptor grade, molecular weight 6,100 daltons with a purity of greater than 98% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), was obtained from Calbiochem Brand Chemicals (San Diego, California). 1,25(OH)2D3 and 25-hydroxyvitamin D3 [25(OH)D3] were generous gifts of Dr. M. Uskokovic, Hoffman La Roche (Nutley, New Jersey).

**Rat Vascular Smooth Muscle Cell Cultures**

The procedure for cultivation of rat VSMC is that of Chamley et al.10 and has been reported in detail previously from our laboratory.11 In brief, segments of aorta from adult Sprague-Dawley rats were stripped of adventitia; then the open ends of the tubes were sealed. The vessels were then incubated in a mixture of 0.05% elastase and 0.1% collagenase for 1–3 hours. Cells obtained in this mixture were pelleted, washed in Hank’s Balanced Salt Solution (HBSS), and dispersed into tissue culture flasks containing Dulbecco’s Modified Eagle Medium (DMEM) that contained 10% fetal calf serum (FCS). At confluence, the cells were released with trypsin
TABLE 1. Measurement by Cell Counts of the Effect of 1,25-Dihydroxyvitamin D₃ on the Growth of Vascular Smooth Muscle Cells Treated With Epidermal Growth Factor

<table>
<thead>
<tr>
<th>Concentration of 1,25(OH)₂D₃ (M)</th>
<th>EGF (5x10⁻¹¹ M)</th>
<th>Cell counts x 10³ (mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1.45 ± 0.09</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>1.76 ± 0.13</td>
</tr>
<tr>
<td>1x10⁻¹¹ M</td>
<td>+</td>
<td>1.32 ± 0.03</td>
</tr>
<tr>
<td>1x10⁻⁹ M</td>
<td>+</td>
<td>1.17 ± 0.02</td>
</tr>
<tr>
<td>1x10⁻⁷ M</td>
<td>+</td>
<td>1.43 ± 0.08</td>
</tr>
</tbody>
</table>

1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; EGF, epidermal growth factor.

0.05%-0.2% EDTA in DMEM and subcultured. Cells were used when homogeneous and after the third passage. Cells obtained by this method have been identified as VSMC by the following criteria: 1) The cells grew in the characteristic hill and valley pattern. 2) They were shown by electron microscopy to possess abundant microfilaments parallel to the long axis of the plasma membrane and to have dense bodies. 3) By immunofluorescence they were reactive to monoclonal antitnyosin antibody (gift of Dr. A Martin, Department of Pharmacology and Cell Biophysics, University of Cincinnati Medical Center, Cincinnati, Ohio) and to monoclonal antitnyosin antibody (gift of Dr. J. Lessard, Children's Hospital Research Foundation, Cincinnati, Ohio).

Assay for Rat Vascular Smooth Muscle Cell Uptake of [³H]Thymidine

An aliquot of 1x10⁴ VSMCs in DMEM that contained 2% FCS was added to wells of flat-bottomed tissue culture plates (Microtest II, Becton Dickinson, Lincoln Park, New Jersey) and cultured with test substances or control media for 48 hours in a 5% CO₂ humidified atmosphere at 37°C. All cultures, including control cultures, were done in 0.04% alcohol, which was required to dissolve the 1,25(OH)₂D₃. An aliquot of 1 μCi [³H]thymidine (20 μCi/mmol) (New England Nuclear, Boston, Massachusetts) was added to each culture for the final 16 hours. At termination of the culture, medium was
FIGURE 3. Graph showing [3H]thymidine uptake of vascular smooth muscle cells (VSMCs) cultured with epidermal growth factor (EGF) alone or a combination of a fixed dose of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] (10⁻⁷ M) and EGF at various concentrations. Values represent mean±SEM of quadruplicate cultures.

For experiments in which we wished to determine if there was a requirement for the concomitant presence of 1,25(OH)₂D₃ to exert its effect, cells were first incubated with 1,25(OH)₂D₃ (10⁻⁷ M) for 72 hours, then thoroughly washed three times to rinse off 1,25(OH)₂D₃, and reincubated with EGF at three concentrations, 1×10⁻¹² M, 5×10⁻¹¹ M, and 2.5×10⁻⁹ M, for another 48 hours. Controls consisted of cells incubated with media for 120 hours, cells incubated with 1,25(OH)₂D₃ for 72 hours, which were then washed and cultured for another 48 hours, and cells cultured with EGF in the final 48 hours of a 120-hour incubation. The cells were pulsed with [³H]thymidine in the final 16 hours of culture. In separate studies, viability of cell preparations at the end of each experimental period was assessed; viability was greater than 95% for cells from both control and experimental cultures.

**Cell Counts**

To verify that the effects observed with the [³H] thymidine assay were reflective of changes in growth, limited experiments were performed in which cell counts were done. In these experiments, 1×10⁵ VSMCs were plated on petri dishes and cultured with various substances (see Table 1) for 96 hours; then the cells were released with 0.5% trypsin-0.02% EDTA and counted with a hemocytometer.

To determine if the effect of 1,25(OH)₂D₃ on EGF-stimulated VSMC growth was mediated by an action on EGF receptor function, studies were done in which the binding of [¹²⁵I]EGF to VSMC was examined with cells that were either untreated or treated with 10⁻⁷ M of 1,25(OH)₂D₃. In these experiments, VSMCs were grown to confluence and then washed twice with phosphate-buffered saline that contained 1 mM CaCl₂, 1 mM MgSO₄, 10 mM HEPES (pH 7.4), and 2 mg/ml bovine serum albumin. Cells were then incubated with various concentrations of [¹²⁵I]EGF-specific activator, 152 μCi/μg (New England Nuclear) for 2 hours (time chosen from preliminary experiments) at room temperature. At the end of the incubation period, the supernatant was pipetted off, and the cells were washed five times with phosphate-buffered saline.
and dissolved in 0.5 ml 0.5N NaDOH. Both supernatants and cell extracts were counted in a gamma counter, and the amount of [125I]EGF bound to the cells was quantitated. Nonspecific binding was determined as cell-associated radioactivity in the presence of a 500-fold excess of unlabeled EGF. The number of cells was determined from the protein content, which was measured by a modification of the method of Lowry et al.12

Table 1 shows the results obtained by cell counting. They show that the cell numbers of cultures treated with 1,25(OH)2D3 were reduced compared with untreated cultures.

Figure 3 shows the results obtained on cell growth when VSMCs were incubated with increasing doses of EGF alone and the effect of combining 1,25(OH)2D3 (constant dose of 10^{-7} M) with the various concentrations of EGF. It is apparent that 1,25(OH)2D3 exercises an inhibitory effect on the response of VSMCs to EGF at all the concentrations tested.

To determine if the concomitant presence of 1,25(OH)2D3 was required for its suppressive effect, experiments were also done in which cells were first incubated with 1,25(OH)2D3 for 72 hours, thoroughly rinsed of 1,25(OH)2D3, then incubated with EGF for another 48 hours. The data (Figure 4) show that when 1,25(OH)2D3 (10^{-7} M) was rinsed off the VSMCs after preincubation for 72 hours, the [3H]thymidine uptake of those cells was equal to those of control cultures, indicating that the presence of 1,25(OH)2D3 was required for its suppressive effect on VSMC growth. Similarly, the proliferative response to EGF (2.5\times10^{-9} M) of cells pretreated with 1,25(OH)2D3 and washed was not different from that of untreated cells, indicating that the continued presence of 1,25(OH)2D3 was required for its action in antagonizing the stimulatory effect of EGF. Similar results were obtained for cells treated with lower concentrations of EGF (data not shown).

Figure 5 shows the binding of [125I]EGF to VSMCs that were either untreated (control) or treated with 1(T7 M 1,25(OH)2D3. No difference in [125I]EGF binding to VSMCs was noted between cells that were either untreated or treated with 1,25(OH)2D3; these observations indicated that 1,25(OH)2D3 had not altered EGF receptor function in VSMCs.

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Discussion
Experiments in the present study demonstrate that 1,25(OH)2D3 inhibits VSMC growth; they also show that the vitamin D metabolite suppresses the mitogenic effects of EGF on VSMC proliferation. There is precedence for such an action of 1,25(OH)2D3 on other cell types. Thus, the substance has been shown to suppress the growth of various cancer cells13-15 as well as the proliferation of immune cells.16

To determine if 1,25(OH)2D3 might exercise its effect on EGF-stimulated VSMCs by an action on EGF receptor function, studies were done in which the binding of [125I]EGF to VSMCs was examined. No differences in the binding of [125I]EGF to VSMCs were observed between cells that were either untreated or treated with 1,25(OH)2D3; these observations indicated that 1,25(OH)2D3 had not altered EGF receptor function in VSMCs.

The results of the current investigation have potential significance to two areas of vascular pathology, namely, hypertension and atherosclerosis. In hypertension, diminished levels of 1,25(OH)2D3 have
been found in the blood of the SHR (compared with the WKY rat) by two groups of investigators using two different techniques. Further evidence of perturbations of 1,25(OH)₂D₃ metabolism in the SHR was recorded by studies that demonstrated a subnormal response of the rat when subjected to stimuli that would raise the level of the hormone. Critically, a recent study showed that in a model of deoxycorticosterone acetate-salt hypertension, manipulations of 1,25(OH)₂D₃ in the diet succeeded in producing a rise of blood pressure when the level of 1,25(OH)₂D₃ was lowered, despite an unchanged level of serum calcium, which suggests a direct effect of the vitamin D metabolite on the blood pressure. The relevance of these observations to human subjects is revealed in studies that have demonstrated subpopulations of patients with abnormal levels of 1,25(OH)₂D₃, including one segment with a diminished level of this substance. The results of our study appear to be germane to the issues involved. Thus, the proliferative rates of VSMCs in the SHR have been demonstrated to be increased compared with the WKY rat, and it may reasonably be speculated that one of the factors responsible for this abnormality is the decreased level of 1,25(OH)₂D₃ found in these rats.

In atherosclerosis, one of the key events in the genesis of the atherosclerotic lesion appears to be VSMC proliferation (reviewed in Reference 20). Among the events leading to such VSMC proliferation may be interactions between infiltrating monocytes and macrophages and VSMCs. Macrophage products have previously been shown to stimulate VSMC proliferation. Recent studies have also shown that activated macrophages elaborate 1,25(OH)₂D₃. Our finding that 1,25(OH)₂D₃ suppresses VSMC growth would suggest that the activated macrophage can secrete both stimulatory and inhibitory factors; the net result on the VSMC would depend on the relative amounts and potencies of the substances produced. At a clinical level, it may be of interest to determine if patients with severe atherosclerosis have any abnormality in 1,25(OH)₂D₃ metabolism. In the clinical syndrome of chronic renal failure, there is a high incidence of atherosclerotic heart disease in such patients, many of whom also have diminished levels of circulating 1,25(OH)₂D₃. Although it is acknowledged that there are many potential causes of atherosclerosis in these patients, such as hypertension and disorders of lipid metabolism, it is entirely reasonable to suggest that this perturbation of 1,25(OH)₂D₃ metabolism may be a contributing factor. Similar consideration may apply to the genesis of hypertension in these subjects.

Acknowledgments

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References


FIGURE 5. Graph showing binding of [125I]EGF to vascular smooth muscle cells. The cells were either untreated (control cells) or incubated with 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] at a concentration of 10⁻⁷ M [1,25(OH)₂D₃-treated cells]. Nonspecific binding was determined as cell-associated radioactivity in the presence of a 500-fold excess of radiolabeled EGF. Values represent mean of triplicate determinations.


KEY WORDS • vitamin D3 • vascular smooth muscle cells • atherosclerosis
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