1,25-Dihydroxyvitamin D_3 Modulates Growth of Vascular Smooth Muscle Cells

Takako Mitsuhashi, R. Curtis Morris, Jr., and Harlan E. Ives
Nephrology Division, Cardiovascular Research Institute and General Clinical Research Center, Department of Medicine, University of California at San Francisco, San Francisco, California 94143

Abstract

We examined the effects of 1,25-dihydroxyvitamin D_3(1,25-$(OH)_2D_3$) on the proliferation of vascular smooth muscle (VSM) cells. Receptors for 1,25-$(OH)_2D_3$ were demonstrated in fresh rabbit aortic tissue and in cultured rat VSM using binding of [^{3}H]-1,25-$(OH)_2D_3$ in sucrose density gradients of the tissue or cell homogenates. The receptor sedimented at 3.6 S, the sedimentation velocity of 1,25-$(OH)_2D_3$ receptors from other sources. 1,25-$(OH)_2D_3$ dramatically altered the growth of VSM, but this effect depended importantly on the basal conditions in which the cells were grown. In quiescent VSM deprived of serum for 72 h, 1,25-$(OH)_2D_3$ (0.1–10 nM), but not 25-$(OH)D_3$ (up to 100 nM) increased thymidine incorporation up to 12-fold and cell number up to 2.6-fold compared with controls. The maximal effect of 1,25-$(OH)_2D_3$ on thymidine incorporation was similar to the maximal effect of the growth factors α-thrombin or PDGF. Furthermore, the effects of 1,25-$(OH)_2D_3$ and thrombin on thymidine incorporation in quiescent cells were markedly synergistic, yielding a 78-fold increase in thymidine incorporation when both agents were added simultaneously. In "nonquiescent cells" which were exposed to serum-free medium for only 24 h, 1,25-$(OH)_2D_3$ (10 nM) also increased DNA synthesis 10-fold compared with controls. However, in striking contrast to what was observed in quiescent cells, 1,25-$(OH)_2D_3$ diminished the mitogenic response to thrombin by as much as 50% in nonquiescent cells. 1,25-$(OH)_2D_3$ also modulated the transcription of c-*myc* in response to thrombin. In quiescent cells, transcription was enhanced by 1,25-$(OH)_2D_3$, whereas in nonquiescent cells, thrombin-induced c-*myc* transcription was blunted. Thus, 1,25-$(OH)_2D_3$ is a potent modulator of the growth of cultured VSM. The direction of this modulation depends strongly on the conditions under which the cells are cultured. (*J. Clin. Invest.* 1991. 87:1889–1895.) Key words: vitamin D_3 • vascular smooth muscle • growth factors • c-*myc*

Introduction

1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$),[1] through its actions in the kidney, intestine, and bone, plays a critical role in Ca^{2+} homeostasis (1–5). Recently, receptors and actions for this hormone have been found in many tissues in addition to these traditional targets, including heart, brain, breast, and many tumor cells (6–13). These novel actions of 1,25-$(OH)_2D_3$ may have significant physiologic, pathologic, and even therapeutic implications. For example, 1,25-$(OH)_2D_3$ induces differentiation of keratinocytes (6) and is useful in the treatment of psoriasis (7). 1,25-$(OH)_2D_3$ also induces differentiation and inhibits DNA synthesis in HL-60 human promyelocytic leukemia cells (8, 9), mouse M1-60 myeloid leukemia cells (10), and human U-937 lymphoma cells (11). On the other hand, 1,25-$(OH)_2D_3$ stimulates the proliferation of circulating monocytes (12) and breast carcinoma cells (13).

1,25-$(OH)_2D_3$ exerts its effects upon cells through the mediation of the vitamin D receptor (VDR), a member of the steroid superfamily of receptors. These receptors are DNA-binding proteins and act by altering the transcription of genes which have the appropriate DNA response elements in their promoters (1–5, 14). For example, 1,25-$(OH)_2D_3$ induces the transcription of genes for calcium binding proteins in intestine (1) and for the osteocalcin gene in bone (14). In other systems, 1,25-$(OH)_2D_3$ may suppress gene expression. In HL-60 cells, Reitsma et al. observed that differentiation was preceded by a decrease in expression of the protooncogene c-*myc* (9). Like the glucocorticoid receptor (15), the VDR may thus be a positive transcription factor for certain genes and a repressor for others.

A variety of experimental work suggests that 1,25-$(OH)_2D_3$ may play a role in certain forms of hypertension. Young spontaneously hypertensive rats and Dahl salt-sensitive rats have higher 1,25-$(OH)_2D_3$ levels than Wistar Kyoto rats and Dahl salt-resistant rats, respectively (16, 17). In humans, it has been reported that serum 1,25-$(OH)_2D_3$ is significantly increased in patients with low renin hypertension (18, 19). Furthermore, decreased dietary Ca^{2+}, which elevates 1,25-$(OH)_2D_3$ levels, has been proposed to contribute to hypertension in some individuals (20, 21). To date, it remains unclear how 1,25-$(OH)_2D_3$ might contribute to the development of hypertension. Receptors for 1,25-$(OH)_2D_3$ have been found in heart (22) and in cultured vascular smooth muscle cells (23). Moreover, 1,25-$(OH)_2D_3$ appears to alter Ca^{2+} metabolism (24) and growth (25, 26) of vascular cells. It was also recently reported that 1,25-$(OH)_2D_3$ is synthesized in vascular endothelial cells (27). Taken together, these findings raise the possibility that 1,25-$(OH)_2D_3$ might have important actions in vascular smooth muscle cells and might play a role in the pathogenesis of atherosclerosis and hypertension.

To further investigate whether there is a role for 1,25-$(OH)_2D_3$ in vascular smooth muscle function, we sought to determine (*a*) whether 1,25-$(OH)_2D_3$ receptors are present in freshly isolated aortic tissue, as they are in cultured vascular smooth muscle (VSM) cells; (*b*) whether 1,25-$(OH)_2D_3$ affects proliferation of this cell type; and (*c*) whether 1,25-$(OH)_2D_3$ affects transcription of oncogenes in VSM. We find that 1,25-$(OH)_2D_3$ is a potent growth factor in vascular smooth muscle

Address reprint requests and correspondence to Harlan E. Ives, M.D., Ph.D., 1065 Health Sciences East, Box 0532, University of California San Francisco, San Francisco, CA 94143.

Received for publication 16 March 1990 and in revised form 17 January 1991.

1. *Abbreviations used in this paper:* 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; TED, Tris/EDTA/dithiothreitol buffer; VDR, vitamin D receptor; VSM, vascular smooth muscle.

J. Clin. Invest.

0021-9738/91/06/1889/07 $2.00
Volume 87, June 1991, 1889–1895

cells and that it modulates the mitogenic action of thrombin and the induction of c-*myc* by thrombin in cultured VSM. These actions of 1,25-$(OH)_2D_3$ in vascular cells may be important in certain pathological states.

Methods

Materials. Unlabeled 1,25-$(OH)_2D_3$ was the generous gift of Dr. M. Uskokovic of Hoffman-LaRoche (Nutley, NJ). 25-$(OH)D_3$ was a generous gift from Dr. Bernard Halloran (University of California, San Francisco). [^{3}H]1,25-$(OH)_2D_3$ (95 Ci/mmol) and [^{32}P]dCTP(400 Ci/mmol, 3,000 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). [^{3}H]Thymidine (6.7 Ci/mmol) and [^{14}C]ovalbumin (0.0136 mCi/mg) were from Dupont Co. (Boston, MA). BSA was fatty acid poor fraction V from Miles Laboratories, Inc. (Naperville, IL). Highly purified human α-thrombin was generously supplied by John W. Fenton II (Albany, NY). PDGF was recombinant homodimeric *sis* product (BB) purchased from Amgen (Thousand Oaks, CA). HL-60 cells were the kind gift of Dr. Masato Mitsuhashi (Howard Hughes Medical Institute, San Francisco, CA). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture. Neonatal rat vascular smooth muscle cells (strain R22D) were obtained from Peter Jones (University of Southern California) (28) and were used for the present studies at passages 16–28. The cells were maintained in Eagle's MEM with 10% FBS, 2% tryptose phosphate broth, penicillin (50 U/ml), and streptomycin (50 U/ml) in a humidified atmosphere of 5% CO_2, 95% air at 37°C (29). Cells were harvested with 3 ml trypsin-versene and 3 ml 0.2% pancreatin and subcultured at a 1:10 dilution weekly. Culture medium was changed every other day until the cells were confluent. Upon achieving confluency, cells to be used as "quiescent" were growth arrested in quiescence medium (MEM containing 20 mM Hepes, 27 mM HCO_3^- (pH 7.4), 5 μg/ml transferrin, 0.5 mg/ml fatty acid free BSA (Miles Laboratories, Inc.), penicillin (50 U/ml), and streptomycin (50 U/ml) and cultured in a humidified atmosphere of 5% CO_2, 95% air at 37°. Quiescence medium was changed daily for 3 d before experimentation. Cells to be used as "nonquiescent" were placed in the same quiescence medium, but for only 24 h before experimentation. HL-60 cells were cultured in suspension as previously described (30).

1,25-$(OH)_2D_3$ receptor binding studies. 1,25-$(OH)_2D_3$ receptors were examined in aortic tissue, HL-60 cells, and cultured VSM cells using previously described methods (22, 23, 31). Aortic tissue was obtained by removing the thoracic aortae from 17 male New Zealand white rabbits (2 kg). After removing all adherent adventitia, the medial strips were washed in 0.9% NaCl and were minced and homogenized with 10 strokes of a Teflon-glass homogenizer driven by a 3/8-in electric drill. HL-60 cells in suspension (2×10^7) were centrifuged at 1,000 rpm in a tabletop centrifuge; rat VSM (3–175 cm^2 flasks; 5×10^6 cells/flask) were suspended as described under "cell culture" and were also centrifuged at 1,000 rpm. Pellets of both cell types were resuspended in TED buffer (10 mM Tris, 1.5 mM EDTA, 10 mM dithiothreitol, pH 7.4) and homogenized with 10–12 strokes in a Teflon-glass handheld homogenizer. Homogenates from each of the three sources were suspended in TED buffer containing Trasylol (500 kallikrein inhibitory U/ml) and PMSF (300 μM) and centrifuged at 4,500 *g* for 10 min. Supernatants were discarded and pellets were washed three times with the same buffer and recentrifuged at 4,500 *g*. These crude nuclear pellets were suspended in 1 ml KTEDMo buffer (TED buffer plus 10 mM sodium molybdate and 0.3 M KCl) with 0.5% Triton X-100 and trasylol and PMSF as above. Then they were centrifuged (200,000 *g* for 1 h) to recover the KCl extracted receptors. 300-μl aliquots of these suspensions were incubated with 1.0 nM [^{3}H]1,25-$(OH)_2D_3$ for 1 h at 4°C in the presence or absence of a 1,000-fold excess of unlabeled 1,25-$(OH)_2D_3$. 200 μl was then layered onto cold 5–20% sucrose gradients (4.8 ml in KTEDMo plus Trasylol and PMSF) and centrifuged (SW55Ti rotor, Beckman) at 200,000 *g* for 24 h. To normalize the results, [^{14}C]ovalbumin (3.6 S) was added to parallel sucrose gradients which were made simultaneously with the sample gradients. 30 fractions were collected; sucrose density was checked on an Abbe refractometer and the samples (140 μl) were counted in a scintillation counter. Specific binding of 1,25-$(OH)_2D_3$ was the difference between total binding and binding in the presence of excess unlabeled ligand.

DNA synthesis and cell number. Cultured VSM cells were harvested, plated onto 24-well plates (16-mm wells), and cultured for 4 d in MEM containing penicillin, streptomycin, and 10% FCS. The confluent monolayers were then placed in quiescence medium (MEM with BSA (0.5 mg/ml), Hepes (20 mM, pH 7.4) and transferrin (5 μg/ml) for an additional 4 d. Cells were then exposed to thrombin (0.4 U/ml), PDGF (10 ng/ml), or 1,25-$(OH)_2D_3$ at various concentrations. After 24 h, cells were incubated with 1 μCi/ml ^{3}H-thymidine for 6 h. Cells were then fixed with 1 ml of 15% TCA. TCA-insoluble counts were measured in a scintillation counter. In separate experiments, cells grown under identical conditions were harvested with 0.05% trypsin and 0.2% pancreatin, and were counted in a Coulter counter (Coulter Electronics, Inc., Hialeah, FL).

Data presentation and statistics. Results are presented as mean±SE for experiments with three or more observations. All comparisons were based on statistical analysis of experiments performed on the same day using the same batch of cells. Comparisons were made using the unpaired Student's *t* test.

Results

Presence of 1,25-$(OH)_2D_3$ receptor (VDR) in aorta and cultured vascular smooth muscle (VSM) cells. To determine whether vitamin D receptors are present in the intact aorta, a nuclear extract of homogenized aortic tissue was incubated with unlabeled and/or labeled 1,25-$(OH)_2D_3$ and subjected to sucrose gradient centrifugation. As shown in Fig. 1, specific binding of the label was observed at a sedimentation velocity of 3.6 S. This value corresponds to the sedimentation velocity of VDR from other sources, such as chick intestine (31), rat heart (22), and cultured HL-60 human leukemia cells (32). Specific binding of [^{3}H]-1,25-$(OH)_2D_3$ in density gradients of nuclear extracts from cultured VSM and HL-60 cells were determined similarly and are shown in Fig. 2. HL-60 cells demonstrated approximately twice as much specific binding as an equal number of VSM cells (1,000 cpm vs. 500 cpm integrated over all active fractions). Thus, both intact aorta and cultured VSM express the 1,25-$(OH)_2D_3$ receptor.

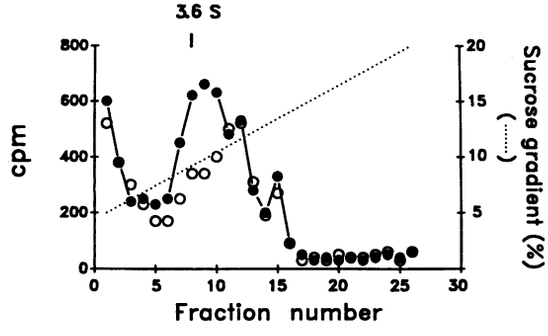

Figure 1. Binding of [^{3}H]1,25-$(OH)_2D_3$ to nuclear extracts of rabbit aorta. Extracts of aortic nuclei were prepared, incubated with labeled and unlabeled 1,25-$(OH)_2D_3$, and subjected to sucrose density gradient (5–20%) separation as described in Methods. Fractions were collected and counted. Total binding of 1,25-$(OH)_2D_3$ (*solid circles*), nonspecific binding (*open circles*), and sucrose density (*dotted line*) are plotted as a function of fraction number. Specific binding was demonstrated in the region of the gradient where the 3.6 S ^{14}C-ovalbumin marker equilibrated in parallel sucrose gradients.

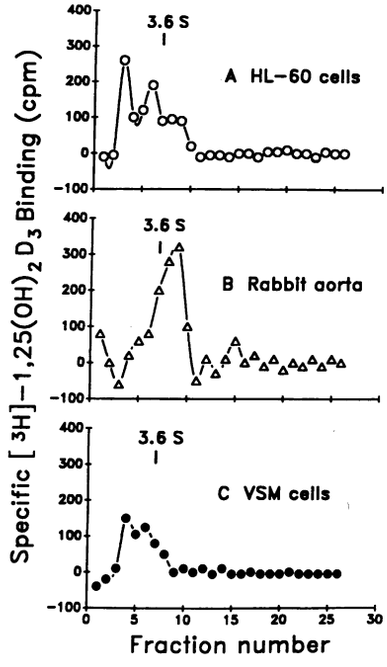

Figure 2. Specific binding of 1,25-$(OH)_2D_3$ in nuclear extracts from HL-60 cells (*A*), rabbit aorta (*B*), and VSM cells (*C*). Nuclear extracts were prepared, incubated with [^{3}H]1,25-$(OH)_2D_3$ as described in Fig. 1 and Methods. Nonspecific binding was subtracted from total binding to obtain specific binding. 3.6 S marks the median fraction of the distribution of ^{14}C-ovalbumin in parallel gradients with identical sucrose density profiles to the tubes containing the nuclear extracts.

Effect of 1,25-$(OH)_2D_3$ on cell proliferation. To investigate the effect of 1,25-$(OH)_2D_3$ on proliferation of VSM, we incubated quiescent VSM with 1,25-$(OH)_2D_3$, thrombin, or PDGF and compared the effects of these various agents on cellular DNA synthesis (Fig. 3, *open bars*). Cells were made quiescent by deprivation of serum for 72 h before addition of growth factors. Under these conditions, 1,25-$(OH)_2D_3$ (10 nM) increased thymidine incorporation into DNA by 12±3 (n = 6)–fold. This stimulation was comparable with that seen by maximal concentrations of other known growth factors, in particular thrombin (0.4 U/ml; 17±4 (n = 6)–fold) and PDGF (10 ng/ml; 14±2 (n = 4)–fold). In contrast to 1,25-$(OH)_2D_3$, 25-$(OH)D_3$ (100 nM) failed to increase thymidine incorporation significantly. The effect of 1,25-$(OH)_2D_3$ was dose-dependent, with a maximal effect at 10 nM (see below).

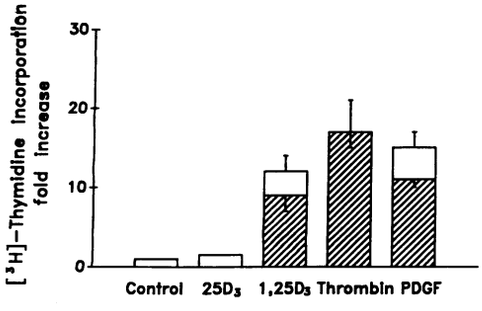

Figure 3. Effect of 1,25-$(OH)_2D_3$, thrombin, and PDGF on DNA synthesis in cultured VSM. As described in Methods, confluent cultures of VSM were deprived of serum for 72 h (quiescent cells, *open bars*) or 24 h (nonquiescent cells, *hatched bars*) and were then incubated with 25-$(OH)D_3$ (100 nM), 1,25-$(OH)_2D_3$ (10 nM), thrombin (0.4 U/ml), or PDGF (10 ng/ml) for 24 h. [^{3}H]Thymidine was then added for 6 h; cells were harvested and TCA insoluble counts were determined as described in Methods. Control (background) thymidine incorporation was 1,155±592 cpm/dish (quiescent cells, *open bars*) and 5,209±1,071 cpm/dish (nonquiescent cells, *hatched bars*). Data is presented as fold increase in [^{3}H]thymidine incorporation over these control values.

To show that the response to 1,25-$(OH)_2D_3$ in quiescent cells was not due to the severe conditions of serum deprivation, the experiments of Fig. 3 were repeated under conditions in which serum was removed for 24 h, rather than 72 h, before addition of growth factors. With this shorter exposure to serum-free medium, basal incorporation of thymidine into DNA is approximately fivefold higher than in quiescent VSM cells. Cells grown under these conditions are therefore referred to as "nonquiescent." Despite the higher basal level of thymidine incorporation in nonquiescent cells, the *relative* increases in thymidine incorporation induced by 1,25-$(OH)_2D_3$, thrombin or PDGF were similar to what was observed with quiescent cells (Fig. 3, *hatched bars*).

To show that thymidine incorporation induced by 1,25-$(OH)_2D_3$ in quiescent cells actually represented increased DNA synthesis and not increased uptake of thymidine from the medium, we compared incorporation of thymidine into soluble (cytosolic) and insoluble fractions after incubation with various concentrations of 1,25-$(OH)_2D_3$ (Fig. 4). 1,25-$(OH)_2D_3$ had no effect upon cytosolic uptake of thymidine, but increased incorporation of thymidine into acid insoluble material significantly. Thus, 1,25-$(OH)_2D_3$ induces incorporation of thymidine into DNA, but does not increase the size of cytosolic pools of thymidine.

1,25-$(OH)_2D_3$ not only caused an increase in DNA synthesis of VSM cells, but also caused the cells to divide (Table I). In quiescent cells, 10 nM 1,25-$(OH)_2D_3$, but not 25-$(OH)D_3$, increased the number of cells 2.6-fold (n = 3, $P < 0.05$) over control dishes not exposed to 1,25-$(OH)_2D_3$ (Table I). Similar to what was observed with DNA synthesis, the effect of 1,25-$(OH)_2D_3$ on cell number was comparable to the effect of 0.4 U/ml thrombin and was dose dependent, with the maximal effect at 10 nM.

Combined effects of 1,25-$(OH)_2D_3$ with thrombin and PDGF. Having found that 1,25-$(OH)_2D_3$ by itself is a potent growth factor, we next asked whether 1,25-$(OH)_2D_3$ affects the cell proliferation induced by other growth factors. MacCarthy et al. reported that in VSM cells grown in 2% serum; prior treatment with 1,25-$(OH)_2D_3$ actually blunted the response to subsequent addition of EGF (26). Similarly, we found in our nonquiescent VSM cells that 48 h pretreatment with 1,25-$(OH)_2D_3$ significantly blunted the response to subsequent addition of either thrombin or PDGF (Fig. 5). Half-maximal inhibition of thrombin-induced mitogenesis by 1,25-$(OH)_2D_3$ was

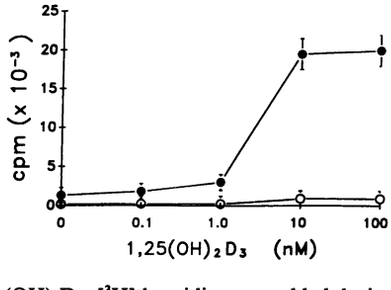

Figure 4. Effect of 1,25-$(OH)_2D_3$ on thymidine uptake and incorporation into DNA in quiescent cells. Confluent, quiescent VSM cells were treated with the indicated concentrations of 1,25-$(OH)_2D_3$ for 24 h. In the continued presence of 1,25-$(OH)_2D_3$, [^{3}H]thymidine was added during an additional 6 h incubation. After removal of the medium and washing with buffer (see Methods), cells were treated with TCA. TCA soluble (*solid circles*) and insoluble (*open circles*) material was separated and counted.

Table I. Effect of 1,25-$(OH)_2D_3$ on Proliferation of VSM

Additive	Cell No.
None	119200±23107
1,25-$(OH)_2D_3$	
1 nM	206693±29517
10 nM	313493±31215*
100 nM	326320±16909*
Thrombin (0.4 U/ml)	430266±35448*

Quiescent cultures of VSM were incubated with the indicated concentrations of 1,25-$(OH)_2D_3$ for 24 h. After trypsinization, the cells were harvested and counted in a Coulter counter. Numbers are mean±SE for triplicate dishes. * $P < 0.05$.

between 0.1 and 1.0 nM (Fig. 6). Thus, whereas 1,25-$(OH)_2D_3$ is a potent growth factor in nonquiescent cells, it may blunt the responses to other growth factors.

Synergistic effect of 1,25-$(OH)_2D_3$ on thrombin-induced mitogenesis in quiescent cells. Surprisingly, the effect of 1,25-$(OH)_2D_3$ (10 nM) on thrombin-induced mitogenesis was quite different in quiescent cells from what was observed in nonquiescent cells. In quiescent cells, 1,25-$(OH)_2D_3$ synergistically increased thymidine incorporation induced by thrombin (from 17±4-fold (thrombin) to 78±10-fold (thrombin plus 1,25-$(OH)_2D_3$) (Fig. 7). With PDGF, 1,25-$(OH)_2D_3$ also enhanced DNA synthesis, but the combined effects of 1,25-$(OH)_2D_3$ and PDGF were only additive. The synergistic effect of 1,25-$(OH)_2D_3$ with thrombin occurred with similar doses of 1,25-$(OH)_2D_3$ to those that alone produced an effect on DNA synthesis (Fig. 8). The half-maximal effect of 1,25-$(OH)_2D_3$ was at ~ 3 nM for both responses.

To further investigate the synergism between 1,25-$(OH)_2D_3$ and thrombin on the growth of quiescent VSM cells, the effect of altering the time between addition of the two ligands was examined (Fig. 9). When 1,25-$(OH)_2D_3$ was added at various times before and after the addition of thrombin, the synergistic effect of the two ligands on DNA synthesis was not as great as when they were added simultaneously. When 1,25-$(OH)_2D_3$ was added 48 h before thrombin, synergism was no longer present. However, 1,25-$(OH)_2D_3$ never blunted the response to thrombin in quiescent cells, as it did in nonquiescent cells (above).

Lastly, we examined the effects of thrombin and 1,25-$(OH)_2D_3$ on expression of the cellular oncogene c-*myc*. Total RNA was isolated from quiescent or nonquiescent VSM cells that had been exposed to thrombin, 1,25-$(OH)_2D_3$, or both. Northern blots were probed with labeled SP6 phage DNA containing the inserted c-*myc* cDNA. In quiescent cells, the effects of thrombin and 1,25-$(OH)_2D_3$ were markedly synergistic (Fig. 10 *a*), whereas in nonquiescent cells 1,25-$(OH)_2D_3$ blocked the induction of c-*myc* message by thrombin. Thus, the effects of 1,25-$(OH)_2D_3$ on transcription of c-*myc* were parallel to its effects on growth of VSM in quiescent and nonquiescent cells.

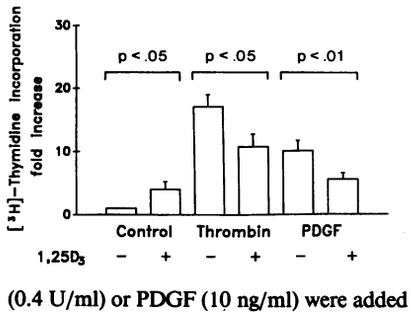

Figure 5. Inhibition of growth factor–induced DNA synthesis by 1,25-$(OH)_2D_3$ in nonquiescent VSM cells. Cultures of VSM were deprived of serum for 24 h. They were then incubated with 10 nM 1,25-$(OH)_2D_3$ or vehicle for 48 h; then thrombin (0.4 U/ml) or PDGF (10 ng/ml) were added for 24 h. [^{3}H]Thymidine was then added for 6 h and the cells were harvested and TCA-insoluble counts determined. Control thymidine incorporation was 4,469±1,046 cpm/dish. Data is presented as fold increase in [^{3}H]-thymidine incorporation over these control values.

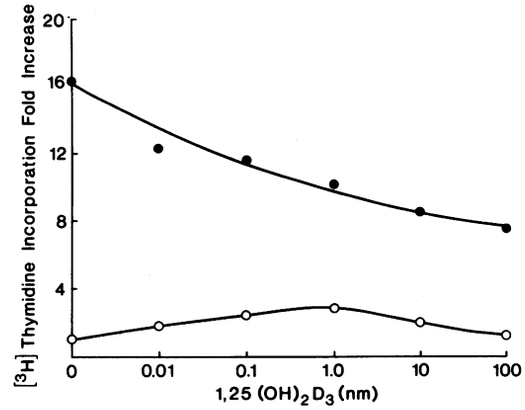

Figure 6. Dose response for inhibition of thrombin-induced DNA synthesis by 1,25-$(OH)_2D_3$ in nonquiescent VSM cells. Nonquiescent cultures of VSM (deprived of serum for 24 h) were incubated with the indicated concentrations of 1,25-$(OH)_2D_3$ for 48 h; thrombin (*solid circles*) or vehicle (*open circles*) was added for 24 h. Thymidine was then added for 6 h and TCA-insoluble counts were determined. Data is plotted as fold increase in thymidine incorporation compared with control cells with no additives. Data shown is a single experiment representative of two similar experiments.

Discussion

Receptors for 1,25-$(OH)_2D_3$ have recently been found in numerous cells and tissues in addition to the traditional targets for

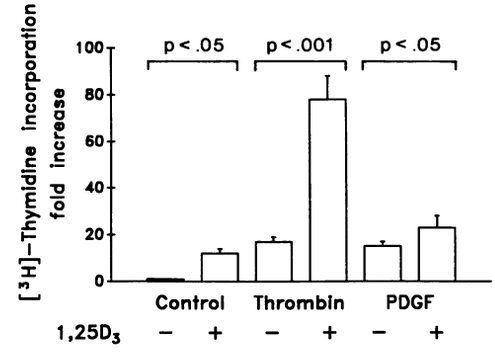

Figure 7. Synergism between 1,25-$(OH)_2D_3$ and thrombin or PDGF on DNA synthesis in quiescent VSM cells. Cultures of VSM were deprived of serum for 72 h. They were then incubated with no additive, thrombin (0.4 U/ml), or PDGF (10 ng/ml), either in the presence or absence of 1,25-$(OH)_2D_3$ for 24 h. [^{3}H]Thymidine was then added for 6 h. Cells were harvested and TCA-insoluble counts determined. Control thymidine incorporation was 1,171±211 cpm/dish. Data is presented as fold increase in [^{3}H]thymidine incorporation over these control values.

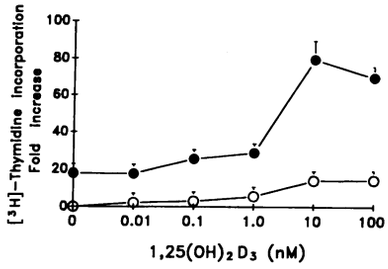

Figure 8. Dose response for stimulation of thrombin-induced DNA synthesis by 1,25-$(OH)_2D_3$ in quiescent VSM cells. Confluent cultures of VSM were deprived of serum for 72 h and were incubated with the indicated concentrations of 1,25-$(OH)_2D_3$ either in the absence (*open circles*) or the presence (*solid circles*) of thrombin (0.4 U/ml) for 24 h. [^{3}H]Thymidine was then added for 6 h, cells were harvested and TCA-insoluble counts were determined. Data is plotted as fold increase in thymidine incorporation compared with control cells with no additives.

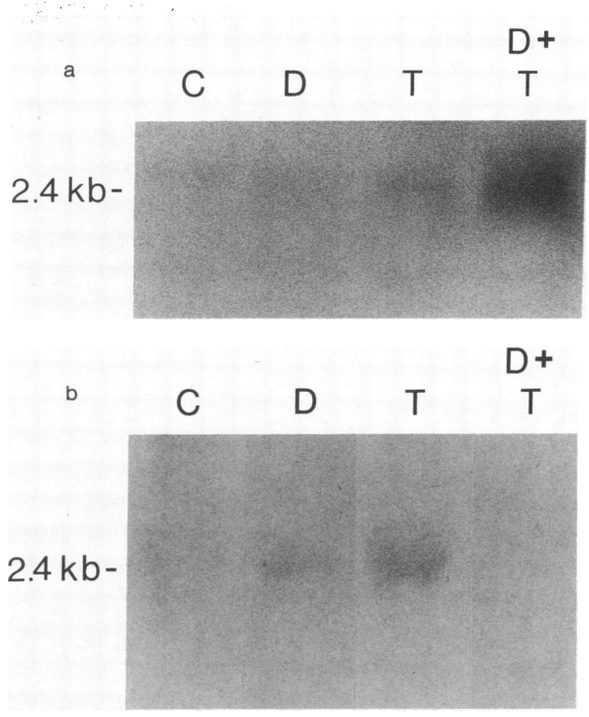

Figure 10. Effect of 1,25-$(OH)_2D_3$ on induction of c-*myc* RNA by thrombin in quiescent and nonquiescent cells. Confluent cultures of VSM were incubated without additives (*c*), 1,25-$(OH)_2D_3$ (*D;* 10 nM), thrombin (*T;* 0.4 U/ml), or both (*D* + *T*). Total cellular RNA was extracted and transferred to nylon filters, then hybridized to c-*myc* or β_2 microglobulin probes as described in Methods. Hybridized blots were washed and autoradiographed. (*a*) Quiescent cells were incubated with ligands for 4 h. (*b*) Nonquiescent cells were incubated with 1,25-$(OH)_2D_3$ for 24 h, then with thrombin for 4 h.

vitamin D action. Among these nontraditional targets are cells from the cardiovascular system, including intact heart (22) and cultured VSM cells (23). The occurrence of vitamin D receptors in the cardiovascular system raises the possibility of an important role for this hormone in normal or disordered cardiovascular physiology. As discussed in the Introduction, a variety of clinical and in vitro data is consistent with a role for 1,25-$(OH)_2D_3$ in the cardiovascular system. Taken together, these disparate findings led us to ask whether 1,25-$(OH)_2D_3$ exerts any biological effects on the VSM cell.

Because most of the biological actions of 1,25-$(OH)_2D_3$ are mediated by a specific receptor, the VDR (2, 3), we first determined whether 1,25-$(OH)_2D_3$ receptors are present in freshly isolated aortic tissue, as well as in cultured VSM cells. In both sources, radiolabeled 1,25-$(OH)_2D_3$ specifically bound to nuclear fractions with a sedimentation coefficient of ~ 3.6 S. Thus, a specific receptor for 1,25-$(OH)_2D_3$ is present both in freshly isolated and cultured VSM cells.

1,25-$(OH)_2D_3$ has been shown to modulate proliferation of several cell types. In most systems where it has been studied, 1,25-$(OH)_2D_3$ reduces the rate of cell proliferation and induces cellular differentiation (see Introduction). We therefore determined whether 1,25-$(OH)_2D_3$ affects the growth rate of VSM cells. We found that the effect of 1,25-$(OH)_2D_3$ depends crucially on the context in which it is added. In nonquiescent cells, 1,25-$(OH)_2D_3$ blunted the growth response to thrombin and PDGF, as well as the induction of c-*myc* RNA by thrombin. On the other hand, in quiescent cells 1,25-$(OH)_2D_3$ enhanced both the growth response and the induction of c-*myc* RNA by thrombin. How can this variable effect of 1,25-$(OH)_2D_3$ be explained? Very recent data on the structure of the vitamin D response element (VDRE) in the promoter of the osteocalcin gene may shed some light on this problem.

Osteocalcin is a major bone protein whose synthesis in osteoblasts is induced by 1,25-$(OH)_2D_3$. The VDRE, found in a region between -513 and -493 bp in the osteocalcin promoter (14), was unexpectedly found to contain the consensus sequence for the AP-1 binding site (33). As for other AP-1 sites, *fos-jun* complexes bind to this site (34). Moreover, when *fos-jun* is bound to the AP-1 site in the osteocalcin promoter, induction of the gene by 1,25-$(OH)_2D_3$ is completely blocked (34). A similar phenomenon has been demonstrated in certain response elements to the glucocorticoid receptor (15). These findings suggest a clear mechanism whereby the response to 1,25-$(OH)_2D_3$ may be "context-dependent." This, or a related phenomenon, may explain why we observed stimulation of DNA synthesis and c-*myc* in quiescent cells, but inhibition of DNA synthesis and c-*myc* in nonquiescent cells that had been treated with thrombin and 1,25-$(OH)_2D_3$. Experiments are currently underway to determine the relative levels of *fos* and *jun* expression under the conditions of the experiments described here.

The effective dose range of 1,25-$(OH)_2D_3$ in the induction of cellular proliferation in quiescent cells was from 10^{-10} to 10^{-8} M (Fig. 8). In nonquiescent cells, inhibition of thrombin-induced mitogenesis was half-maximal at 10^{-10} M, with detect-

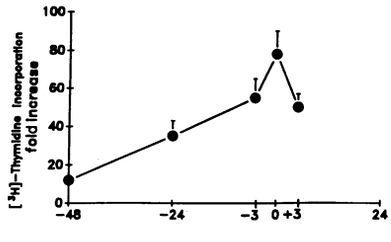

Figure 9. Effect on DNA synthesis of varying the time between addition of thrombin and 1,25-$(OH)_2D_3$. Confluent cultures of VSM were deprived of serum for 72 h (quiescent) and were incubated with thrombin (0.4 U/ml) or vehicle for 24 h as described in Methods. 10 nM 1,25-$(OH)_2D_3$ was added at various times before (negative values) or after (positive values) addition of thrombin. Data is presented as fold increase in thymidine incorporation compared with cells that were treated with neither thrombin nor 1,25-$(OH)_2D_3$. Thrombin alone caused a 15-fold increase in DNA synthesis.

able inhibition observed at 10^{-11} M. This is the same dose range at which 1,25-$(OH)_2D_3$ induces differentiation of mouse myeloid leukemia cells (10) and HL-60 cells (8, 9). More importantly, this dose range is comparable with the concentrations of 1,25-$(OH)_2D_3$ which induce a variety of accepted physiologic responses when carried out with cells and tissues in vitro. For example, half-maximal suppression of transcription of the preproPTH message by 1,25-$(OH)_2D_3$ occurred at 10^{-9} M in freshly isolated parathyroid cells (35); induction of transcription of the osteocalcin gene was half maximal at 10^{-10} M in bone cells (33). Furthermore, the K_d for the vitamin D receptor in HL-60 cells is 5×10^{-9} M (32) and in chick intestine is 3×10^{-10} M (31). Thus, our data indicates that 1,25-$(OH)_2D_3$ exerts biological effects in vascular smooth muscle cells at concentration near the K_d for the receptor and at concentrations similar to that observed for biological effects of vitamin D when measured in many tissues and cell types in vitro. Of note, the total circulating level of 1,25-$(OH)_2D_3$ is 10^{-10} M (36). However, most of this 1,25-$(OH)_2D_3$ is bound to various plasma proteins, leading to a free circulating level of 4×10^{-13} M (36). Because physiological responses to vitamin D have rarely been found at subpicomolar levels when measured in vitro, it is possible that bound vitamin D in the plasma may play some role in the cellular uptake or the response to 1,25-$(OH)_2D_3$ in vivo. This bound vitamin D would not be available in vitro, possibly raising the concentrations needed to elicit responses.

Alternatively, our in vitro system may simply be less responsive to 1,25-$(OH)_2D_3$ than intact vascular smooth muscle cells, perhaps due to alterations in receptor number in the cultured cells. Another interesting possibility is that local concentrations of 1,25-$(OH)_2D_3$ in vascular tissue are higher than free circulating levels of the hormone. Support for this idea comes from the recent observation that vascular endothelial cells actually synthesize 1,25-$(OH)_2D_3$ (27). Lastly, it is possible that the concentrations of 1,25-$(OH)_2D_3$ we used to elicit growth responses are never actually seen by VSM cells in vivo and the effects we observed are due to cross-reaction with other receptors of the steroid superfamily. If so, the cross-reacting receptor is unlikely to be the glucocorticoid receptor, because dexamethasone suppresses growth of VSM (25) under conditions where we found that 1,25-$(OH)_2D_3$ induced growth.

The results of the current study do not yet establish a major role for 1,25-$(OH)_2D_3$ in normal or deranged cardiovascular physiology. However, when viewed in the context of previous data on levels of 1,25-$(OH)_2D_3$ in hypertensive subjects and on the possible role of dietary calcium intake as a determinant of blood pressure, it suggests that under certain conditions 1,25-$(OH)_2D_3$ may exert physiologically important effects on the proliferation of vascular cells.

Acknowledgments

We thank Dr. Floyd C. Rector for his support, Dr. Robert H. Weiss for his helpful comments and discussion, and John Lowe for his superb technical assistance.

This work was supported by National Institutes of Health grants DK34127, HL41210, AM32631, gifts from the Church and Dwight Corp., and funds from the National Dairy Board (administered in cooperation with the National Dairy Council.) H. E. Ives is an established investigator of the American Heart Association.

References

1. Norman, A. W., J. Roth, and L. Orci. 1982. The vitamin D endocrine system: steroid metabolism, hormone receptors and biological response (calcium binding protein). *Endocr. Rev.* 3:331–366.

2. Haussler, M. R. 1986. Vitamin D receptors: nature and function. *Annu. Rev. Nutr.* 6:527–562.

3. Minghetti, P. P., and A. W. Norman. 1988. 1,25-(OH)2-Vitamin D_3 receptors: gene regulation and genetic circuitry. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 2:3043–3053.

4. DeLuca, H. F. 1988. The vitamin D story: a collaborative effort of basic science and clinical medicine. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 2:224–236.

5. Reichel, H., H. P. Koeffler, and A. W. Norman. 1989. The role of the vitamin D endocrine system in health and disease. *N. Engl. J. Med.* 320:980–991.

6. Kuroki, T. 1985. Possible functions of 1,25-dihydroxyvitamin D_3, an active form of vitamin D_3, in the differentiation and development of skin. *J. Invest. Dermatol.* 84:459–460.

7. Holick, M. F., E. Smith, and S. Pincus. 1987. Skin as the site of vitamin D synthesis and target tissue for 1,25-dihydroxyvitamin D_3: use of calcitriol (1,25-dihydroxyvitamin D_3) for treatment of psoriasis. *Arch. Dermatol.* 123:1677–1683.

8. Simpson, R. U., T. Hsu, D. A. Begley, B. S. Mitchell, and B. N. Alizadeh. 1987. Transcriptional regulation of the c-myc protooncogene by 1,25-dihydroxyvitamin D_3 in HL-60 promyelocytic leukemia cells. *J. Biol. Chem.* 262:4104–4108.

9. Reitsma, P. H., P. G. Rothberg, S. M. Astrin, J. Trial, Z. Bar-Shavit, A. Hall, S. L. Teitelbaum, and A. J. Kahn. 1983. Regulation of myc gene expression in HL-60 leukaemia cells by a vitamin D metabolite. *Nature (Lond.).* 306:492–494.

10. Abe, E., C. Miyaura, H. Sakagami, M. Takeda, K. Konno, T. Yamazaki, S. Yoshiki, and T. Suda. 1981. Differentiation of mouse myeloid leukemia cells induced by 1,25-dihydroxyvitamin D_3. *Proc. Natl. Acad. Sci. USA.* 78:4990–4994.

11. Karmali, R., A. K. Bhalla, S. M. Farrow, M. M. Williams, S. Lal, P. M. Lydyard, and J. L. O'Riordan. 1989. Early regulation of c-myc RNA by 1,25-dihydroxyvitamin D_3 in human myelomonocytic U937 cells. *J. Mol. Endocrinol.* 3:43–48.

12. Ohta, M., T. Okabe, K. Ozawa, A. Urabe, and F. Takaku. 1985. 1,25-Dihydroxyvitamin D_3 (calcitriol) stimulates proliferation of human circulating monocytes in vitro. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 185:9–13.

13. Freake, H. C., C. Marcocci, J. Iwasaki, and MacIntyre. 1981. 1,25-Dihydroxyvitamin D_3 specifically binds to a human breast cancer cell line (T470) and stimulates growth. *Biochem. Biophys. Res. Commun.* 101:1131–1138.

14. Kerner, S. A., R. A. Scott, and J. W. Pike. 1989. Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D_3. *Proc. Natl. Acad. Sci. USA.* 86:4455–4459.

15. Diamond, M. I., J. N. Miner, S. K. Yoshinaga, and K. R. Yamamoto. 1990. Transcription factor interactions: selectors of positive or negative regulation form a single DNA element. *Science (Wash. DC).* 249:1266–1272.

16. Lau, K., and B. Eby. 1985. The role of calcium in genetic hypertension. *Hypertension (Dallas).* 7:657–667.

17. Kotchen, T. A., C. E. Ott, and S. A. Whitescarver. 1989. Calcium and calcium regulating hormones in the prehypertensive Dahl salt-sensitive rat. *Am. J. Hypertens.* 2:749–753.

18. Resnick, L. M., F. B. Muller, and J. H. Laragh. 1986. Calcium regulating hormones in essential hypertension: relation to plasma renin activity and sodium metabolism. *Ann. Intern. Med.* 105:649–654.

19. Resnick, L. M., J. P. Nicholson, and J. H. Laragh. 1986. Calcium metabolism in essential hypertension: relationship to altered renin system activity. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 45:2739–2745.

20. McCarron, D. A., C. D. Morris, and C. Cole. 1982. Dietary calcium in human hypertension. *Science (Wash. DC).* 217:267–269.

21. McCarron, D. A., and C. D. Morris. 1987. The calcium deficiency hypothesis of hypertension. *Ann. Int. Med.* 107:919–922.

22. Walters, M. R., D. C. Wicker, and P. C. Riggle. 1986. 1,25-Dihydroxyvitamin D_3 receptors identified in the rat heart. *J. Mol. Cell. Cardiol.* 18:67–72.

23. Merke, J., W. Hoffman, D. Goldschmidt, and E. Ritz. 1987. Demonstration of 1,25-$(OH)_2$ vitamin D_3 receptors and actions in vascular smooth muscle cells in vitro. *Calcif. Tissue Int.* 41:112–114.

24. Bukoski, R. D., H. Xue, and D. A. McCarron. 1987. Effect of 1,25-$(OH)_2$ vitamin D_3 and ionized Ca^{2+} on $^{45}Ca^{2+}$ uptake by primary cultures of aortic myocytes of spontaneously hypertensive and Wistar Kyoto normotensive rats. *Biochem. Biophys. Res. Commun.* 146:1330–1335.

25. Koh, E., S. Morimoto, K. Fukuo, K. Itoh, T. Hironaka, T. Shiraishi, T. Onishi, and Y. Kumahara. 1988. 1,25-Dihydroxyvitamin D_3 binds specifically to rat vascular smooth muscle cells and stimulates their proliferation in vitro. *Life Sci.* 42:215–223.

26. MacCarthy, E. P., W. Yamashita, A. Hsu, and B. S. Ooi. 1989. 1,25-Dihy-

droxyvitamin D_3 and rat vascular smooth muscle cell growth. *Hypertension (Dallas).* 13:954–959.

27. Merke, J., P. Milde, S. Lewicka, U. Hugel, G. Klaus, D. J. Mangelsdorf, M. R. Haussler, E. W. Rauterberg, and E. Ritz. 1989. Identification and regulation of 1,25-dihydroxyvitamin D_3 receptor activity and biosynthesis of 1,25-dihydroxyvitamin D_3. *J. Clin. Invest.* 83:1903–1915.

28. Jones, P. A., T. Scott-Burden, and W. Gevers. 1979. Glycoprotein, elastin, and collagen secretion by rat smooth muscle cells. *Proc. Natl. Acad. Sci. USA.* 76:353–357.

29. Mitsuhashi, T., and H. E. Ives. 1988. Intracellular Ca requirement for activation of the Na^+/H^+ exchanger in vascular smooth muscle cells. *J. Biol. Chem.* 263:8790–8795.

30. Mitsuhashi, M., T. Mitsuhashi, and D. G. Payan. 1989. Multiple signaling pathways of histamine H2 receptors. *J. Biol. Chem.* 264:18356–18362.

31. Brumbaugh, P. F., and M. R. Haussler. 1975. Specific binding of 1,25-dihydroxycholecalciferol to nuclear components of chick intestine. *J. Biol. Chem.* 250:1588–1594.

32. Mangelsdorf, D. J., P. Koeffler, C. A. Donaldson, J. W. Pike, and M. R. Haussler. 1984. 1,25-Dihydroxyvitamin D_3-induced differentiation in human promyelocytic leukemia cell line (HL-60): receptor-mediated maturation to macrophage-like cells. *J. Cell Biol.* 98:391–398.

33. Morrison, N. A., J. Shine, J.-C. Fragonas, V. Verkest, L. McMenemy, and J. A. Eisman. 1989. 1,25-Dihydroxyvitamin D-responsive element and glucocorticoid repression in the osteocalcin gene. *Science (Wash. DC).* 246:1158–1161.

34. Schule, R., K. Umesono, D. J. Mangelsdorf, J. Bolado, J. W. Pike, and R. M. Evans. 1990. Jun-fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene. *Cell.* 61:497–504.

35. Silver, J., J. Russell, and L. M. Sherwood. 1985. Regulation by vitamin D metabolites of messenger ribonucleic acid for preproparathyroid hormone in isolated bovine parathyroid cells. *Proc. Natl. Acad. Sci. USA.* 82:4270–4273.

36. Bikle, D. D., E. Gee, B. Halloran, and J. G. Haddad. 1984. Free 1,25-dihydroxyvitamin D levels in serum from normal subjects, pregnant subjects, and subjects with liver disease. *J. Clin. Invest.* 74:1966–1971.