1,25-Dihydroxyvitamin D_3 and the Regulation of Human Cancer Cell Replication[1] (42912)

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Abstract. **Several human and animal cancer cell lines have been shown to possess specific high affinity receptors for 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$). The replication of several of these cell types has also been shown to be regulated by this hormone, both *in vitro* and *in vivo*. To further understand the mechanisms of these actions, we have examined cancer cells *in vitro* and *in vivo*. The *in vitro* studies extend our previous reports on the treatment of human breast cancer cells (T 47D) with 10^{-9} to 10^{-6} *M* 1,25-$(OH)_2D_3$, which resulted in a dose- and time-dependent decrease in cell numbers over 6 days. Treatment with 10^{-8} *M* 1,25-$(OH)_2D_3$, which reduced cell numbers to approximately one half of those found in control cultures at 6 days, was associated with a doubling of the proportion of cells in the G_2 + M phase of the cell cycle and was accompanied by a significant decline in the proportion of G_0/G_1 cells. At higher concentrations there was a significant decline in S phase cells with accumulation of cells in both G_0/G_1 and G_2 + M phases. The antiestrogen, tamoxifen, at a concentration which caused similar effects on cell number, resulted in proportional decreases in both S and G_2 + M phase cells and accumulation of G_0/G_1 cells. The effects of 1,25-$(OH)_2D_3$ on T 47D cell proliferation were associated with time- and concentration-dependent reductions in epidermal growth factor receptor levels to a minimum level of about half that seen in control cultures. The *in vivo* experiments extend our previous studies, which demonstrated marked inhibition of the growth of human cancer xenografts in immunosuppressed mice by 1,25-$(OH)_2D_3$. Xenograft growth was inhibited with 1,25-$(OH)_2D_3$ (0.1 μg ip three times per week) but growth was rapidly restored when the 1,25-$(OH)_2D_3$ was withdrawn. Thus, there are clear-cut time- and dose-dependent, yet reversible, effects of 1,25-$(OH)_2D_3$ on the replication of human cancer cells *in vitro* and *in vivo*, which are possibly mediated through changes in growth factor receptor levels. Further study of these effects may advance understanding of the hormonal control of cellular replication in human cancers.**

[P.S.E.B.M. 1989, Vol 191]

Vitamin D has long been studied in relation to bone and calcium homeostasis. It has been shown that the vitamin is progressively metabolized by the liver and then the kidney to an active hormonal form, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$). Recent studies however have led to the realization that this hormone has a quite distinct and previously unsuspected role in the regulation of cellular replication (1–8). These studies have been carried out in several human and animal cancer cell lines. The work presented here relates to studies in human breast cancer, colonic cancer, and malignant melanoma cells. The dose response of 1,25-$(OH)_2D_3$ in human breast cancer cells *in vitro* closely resembles those of estrogens on the replication of these cells. Thus, 1,25-$(OH)_2D_3$ at low concentrations (10^{-12} to 10^{-10} *M*) stimulates and at high concentrations (>10^{-9} *M*) inhibits cellular replication (4–7, 9). Although these cancer cell lines possess specific, high affinity receptors for 1,25-$(OH)_2D_3$, the cellular mechanisms by which the effects on replication are mediated are not understood. Recent *in vitro* studies have demonstrated that human breast cancers secrete polypeptide growth factors and that the secretion of these polypeptides is affected by estrogens (10, 11). Furthermore, these same breast cancer cells possess growth factor receptors, including those for epidermal growth factor (EGF) and the latter can be regulated by progestins (11–13). Estrogens, progestins, and 1,25-$(OH)_2D_3$ all affect the replication of the same cell types, which in the cases of the former two steroids are associated with distinctive changes in cell cycle kinetics. We

[1] Some of the material in this article was presented at the Seventh Vitamin D Workshop, Rancho Mirage, California, April 1988.

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0037-9727/89/1913-0221$2.00/0
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have therefore examined the effects of 1,25-$(OH)_2D_3$ on both cell cycle kinetic parameters and the EGF receptor levels. The effects of 1,25-$(OH)_2D_3$ on cellular replication *in vitro* have already been confirmed *in vivo*; 1,25-$(OH)_2D_3$ in high doses prolongs the survival of mice injected with syngeneic leukemia cells (14) and inhibits the growth of human malignant melanoma and colonic cancer cell xenografts in immunosuppressed mice (15). Hence, 1,25-$(OH)_2D_3$, which has receptors in a wide variety of human cancer cell lines from various tissues of origin, may have the potential for treatment of human malignancy analogous to that of estrogens and antiestrogens. This application requires that the obvious clinical limitation of hypercalcemia be overcome. With respect to this latter problem, we have initiated studies into dosage regimens, which could limit hypercalcemia.

Materials and Methods

In vitro studies were performed with T 47D human breast cancer cells, initially obtained from the EG & G Mason Research Institute, Worchester, MA. Cells were grown in RPMI 1640 medium (Flow) supplemented with 5% fetal calf serum (FCS; Grand Island Biological Co., Sydney, Australia), 200 microunits/ml porcine insulin (CSL-NOVO, Sydney, Australia), 4 m*M* L-glutamine, 14 m*M* sodium bicarbonate, 20 μg/ml each of penicillin and gentamicin, and 20 m*M* Hepes buffer. Cell cycle kinetic studies were performed on cells in exponential growth phase. Cells were passaged by trypsinization with 0.05% trypsin in phosphate-buffered saline (CSL, Sydney, Australia) containing 0.02% disodium EDTA (Flow Laboratories, Sydney, Australia).

All vitamin D_3 metabolites and analogues were gifts from Dr. M. Uskokovic, Hoffmann-La Roche, Nutley, NJ. For the *in vitro* studies 1,25-$(OH)_2D_3$ was added to final concentrations of 10^{-10} to 10^{-6} *M*, the final ethanol (vehicle) concentration was always less than 0.1%. Cells were exposed to the hormone for a period of up to 6 days with the media being changed at the start of the hormone treatment period and again on the third and fifth days. In some studies the 1,25-$(OH)_2D_3$ was added at the start of the incubation period only and in others was replenished at the time of media changes throughout the incubation period. In parallel experiments the antiestrogen tamoxifen was added at 2×10^{-6} *M*, a dose which causes a similar degree of inhibition of cellular replication (16–19) to that seen with the higher concentrations of 1,25-$(OH)_2D_3$.

Cell numbers were determined at various times after drug administration using a Coulter Counter (model Dn; Coulter Electronics, Luton, UK). Cell cycle phase distribution was determined by analytical DNA flow cytometry. Approximately 10^6 cells were stained with ethidium bromide-mithramycin as previously described (20) and treated with 1 mg/ml ribonuclease (Type 1A; Sigma) for 5 to 15 min before analysis on an ICP22 pulse cytometer (Ortho Instruments, Westwood, MA). Excitation was at 360–460 nm and fluorescence detection at greater than 550 nm. Estimates of the proportions of cells in the G_0/G_1, S, and G_2 + M phases of the cell cycle were calculated from the DNA histograms using a planimetric method of analysis (21). In some studies the effect of 1,25-$(OH)_2D_3$ on the rate of efflux of cells from G_0/G_1 phase was estimated using the cytokinesis inhibitor, ICRF 159 (Razoxone; ICI, Macclesfield, UK). Cells, after treatment with hormone, were exposed to 100 μg/ml ICRF 150 and the efflux from G_0/G_1, corrected for those cells with DNA contents greater than G_2 + M, was followed by DNA flow cytometry as previously described (16).

Receptor studies for EGF were modified from previously described methods (12, 13). Briefly, cells were plated into 24-well tissue culture trays and grown to confluence. Prior to study the medium was removed and replaced with fresh RPMI 1640 medium containing 1% charcoal-treated FCS and various concentrations of vitamin D_3 metabolites in ethanol (final ethanol concentration 0.1% or less). At the end of 24-hr exposure to the vitamin D_3 compounds, the medium was removed and the cells were washed twice with RPMI 1640 medium without any FCS but supplemented with 0.1% bovine serum albumin. Specific binding of EGF was estimated from incubations with ^{125}I-EGF (~40,000 cpm/well) with or without 50 n*M* unlabeled EGF. At the end of these incubations, which were for 1 hr at 37°C, the medium was removed and the cell monolayers were washed carefully, solubilized, and counted (12, 13).

The effect of 1,25-$(OH)_2D_3$ on the growth of xenografted tumors of the COLO 206F colonic cancer cell line, obtained initially from Dr. George Moore, Denver, Colorado, was studied in immunosuppressed mice weighing 20–25 g, as described previously (15). The mice, maintained on a low calcium (0.1%) soybean flour-based diet, were treated with second daily intraperitoneal injections of 0.05 ml corn oil vehicle (control) or 1,25-$(OH)_2D_3$ (0.1 μg). Immunosuppression of male and female CBA/lac mice was achieved as previously described (22–24). Briefly, mice were thymectomized at 16–18 days of age. After an additional 18–21 days an intraperitoneal injection of 200 mg/kg cytosine arabinoside (Cytostar, Upjohn Co.) was given and followed 48 hr later by 8.5 Gray whole-body irradiation using a ^{137}Cs Grammacell irradiation device (Atomic Energy of Canada Ltd, Ottawa, Canada). Animals were used as xenograft hosts within 4 weeks of irradiation (22–24). Cultures of COLO 206F cells were grown to near confluence in monolayer culture in RPMI 1640 medium supplemented with 5% fetal calf serum. The cells were harvested mechanically using a rubber scraper, washed in serum-free medium, resuspended, and injected subcutaneously (10^5–10^6 cells/site) into each flank of the immunosuppressed mice. Successful

xenografts obtained in the first group of immunosuppressed mice were removed, minced, and passaged in further immunosuppressed mice to establish stable xenografted tumors. After xenografts had grown to a diameter of approximately 1 cm, the greatest and least diameters were measured using Vernier calipers and tumor volumes were estimated using the formula $1/6\ \pi d^3$ where d is the mean of these two diameters. At this time equal numbers of male and female mice were randomly assigned to experimental and control groups with each group containing 10–14 xenografts. Treatment was then started and the tumor volumes estimated every second or third day subsequently. The volume of each tumor on each day of assessment (V_t) was divided by its initial volume (V_o) to obtain the relative tumor volume (V_t/V_o), which was plotted against time and analyzed using the method of least squares of a log-linear regression. Regression coefficients of treated and control xenograft growth were compared by analysis of variance using a MINITAB statistical package.

Results

The inhibition of cell replication by 1,25-$(OH)_2D_3$ was dose dependent in the concentration range of 10^{-9}–10^{-6} M. At the highest dose of 10^{-6} M 1,25-$(OH)_2D_3$ cell numbers were reduced to about 3% of those of cells in control flasks after 6 days of treatment. The inhibitory concentration of 10^{-8} M, which caused an approximate halving of cell numbers with multiple dosing, was not associated with any evidence of cell death. These data support the view that 1,25-$(OH)_2D_3$ decreases T 47D cell numbers by inhibiting the rate of cellular proliferation. Treatment with 1,25-$(OH)_2D_3$ also resulted in concentration- and time-dependent changes in cell cycle parameters (Fig. 1). An increase in G_2 + M phase cells was seen with all concentrations of 1,25-$(OH)_2D_3$ tested. The concentration of 10^{-8} M 1,25-$(OH)_2D_3$, which reduced cell numbers to about one half of that in control cultures, resulted in an approximate doubling of the proportion of cells in G_2 + M phase from 9.7% to 19.6%, significant by paired analysis ($P < 0.002$). This was accompanied by a small decrease in the number of cells in G_0/G_1 from 71% to 63%. At the highest concentration of 1,25-$(OH)_2D_3$, this increase in G_2 + M phase cells was accompanied by an increase in G_0/G_1 phase cells and a virtual emptying out of S phase. Treatment with tamoxifen resulted in an accumulation of cells in G_0/G_1 without any increase, but in fact a decrease, in G_2 + M phase cells.

The increase in G_2 + M phase cells after exposure to 1,25-$(OH)_2D_3$ was dose dependent. The effect was first seen after 3 days of exposure and persisted throughout the 6-day treatment period. G_0/G_1 exit kinetics were not affected by moderately high concentrations ($\geq 5 \times 10^{-8}$ M) of 1,25-$(OH)_2D_3$ but were delayed after treatment with 10^{-7} M 1,25-$(OH)_2D_3$; the $t_{1/2}$ of exit being prolonged approximately 7-fold in the 1,25-$(OH)_2D_3$-treated cells as compared with control cultures.

Incubation of the T 47D cells with 1,25-$(OH)_2D_3$ resulted in a dose-and time-dependent reduction in EGF receptor levels, as we have previously shown (25). This effect, which resulted in a >50% reduction in apparent receptor numbers as determined by Scatchard analysis, was half-maximal at approximately 2 nM 1,25-$(OH)_2D_3$. The effects of different vitamin D_3 metabolites were correlated with their affinities for the 1,25-$(OH)_2D_3$ receptor in that 1-hydroxylated compounds were more potent that those lacking the 1-hydroxyl group (Fig. 2). This supports the view that the effect on the EGF receptor is mediated through the 1,25-$(OH)_2D_3$ receptor.

For the colon carcinoma, COLO 206F, xenografts, volume doubling time in control mice was 7 days. The growth of these xenografts was markedly inhibited by treatment with 1,25-$(OH)_2D_3$ over the entire treatment period as we have shown previously (15). However, when the 1,25-$(OH)_2D_3$ treatment was ceased, the xenografts recommenced growth with a doubling time not longer than that of the untreated control xenografts (Fig. 3).

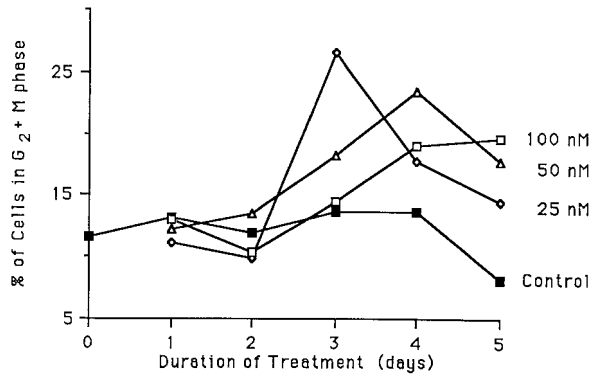

Figure 1. Time course of inhibition concentrations of 1,25-$(OH)_2D_3$ on proportion of cells in G_2 + M phase. Cells were treated with ethanol vehicle or 1,25-$(OH)_2D_3$ at the concentrations indicated.

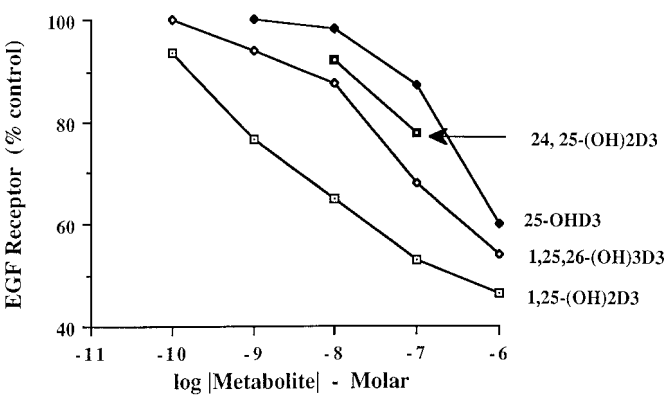

Figure 2. Specificity of effects of vitamin D_3 metabolites on EGF receptor levels.

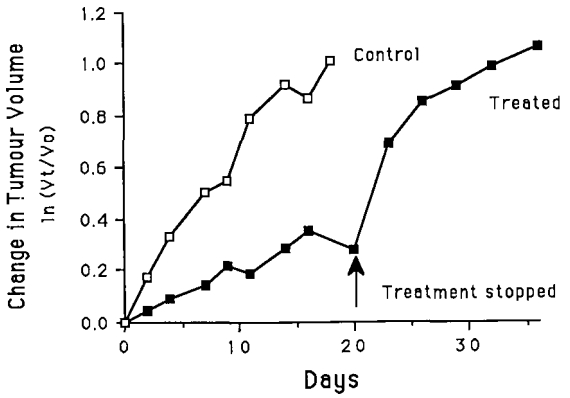

Figure 3. Effect of 1,25-$(OH)_2D_3$ on the growth of the human colonic cancer cell line (COLO 206F) xenografts in immunosuppressed mice. Groups of animals received intraperitoneal injections 3 days per week for 3 weeks of either 0.2 ml of corn oil vehicle (control) or 0.1 μg of 1,25-$(OH)_2D_3$ in corn oil. Tumour volumes were measured at times indicated.

Discussion

It is of considerable interest that all of the inhibitory concentrations of 1,25-$(OH)_2D_3$ tested caused an accumulation of cells in the G_2 + M phase of the cell cycle. This suggests that a primary effect of 1,25-$(OH)_2D_3$ on breast cancer cell replication may be mediated by a transition delay through G_2 or mitosis. At higher concentrations of 1,25-$(OH)_2D_3$, where the inhibitory effects on replication are maximal, increases in both G_2 + M and G_0/G_1 transit times contribute to the overall increase in cell cycle length and a decreased proliferation rate. Indeed, at the highest concentrations tested, virtually no cells appear to remain in S phase, indicating that progression out of G_1 phase was completely blocked, although progression through S phase can proceed. Explanation of these data necessitate differential dose responsiveness for 1,25-$(OH)_2D_3$ effects in G_0/G_1 and G_2 + M phases. The data presented in Figure 1, showing that the magnitude of accumulation in G_2 + M was maximal at the lower concentration of 1,25-$(OH)_2D_3$, suggest that the G_2 + M transition delay is more sensitive to 1,25-$(OH)_2D_3$ than the effect on G_0/G_1 transition. Thus, the more modest effects of higher concentrations of 1,25-$(OH)_2D_3$ on the proportion of G_2 + M phase cells may be accounted for by the fact that cells are unable to reach G_2 + M due to the delay and/or arrest in G_0/G_1 phase. Recent studies of the effects of 1,25-$(OH)_2D_3$ in cells of the hemopoietic lineage have shown accumulation of cells in G_0/G_1 and a block of progression of cells from the early, low RNA to the late, higher RNA compartment of G_1 (26, 27). These observations are consistent with the effects we have reported here with the highest dose of 1,25-$(OH)_2D_3$ in this human breast cancer cell line. They further support the idea of a distinct mechanism of regulation of cell cycle progression and substantial differences in the dose responses of the two major 1,25-$(OH)_2D_3$ inhibitory effects, i.e., on progression through G_1 and through G_2 + M. These effects, distinct from the effects seen with tamoxifen where the predominant effect is to block the progression of cells through G_1 phase (16–19), are being studied further. A small number of other studies from our own and other groups have also shown an accumulation of cells in G_2 + M phase accompanying growth inhibition associated with continuous exposure to tamoxifen (28), high concentrations of estrogen (29), treatment with phorbol ester (30), or very low (0.25%) concentrations of FCS (31).

In this regard, in another 1,25-$(OH)_2D_3$-responsive human cancer cell (HL-60), 1,25-$(OH)_2D_3$ at high concentration (10^{-9} to 10^{-7} M) has been shown to stimulate the production (transiently) of the c-*fos* oncogene and subsequently to inhibit the production of c-*myc* mRNA (32, 33). Hence, it is possible that 1,25-$(OH)_2D_3$ may exert some of its effects in these cells by the regulation of the transcription and/or translation of c-*myc* or other oncogene products. Alternately, autocrine growth factor production may be involved since proliferation rates and cell cycle kinetic parameters in the serum depletion studies (31) were partially restored by addition of growth factors. Furthermore, the data presented here and our recent studies (25) show that 1,25-$(OH)_2D_3$ treatment down-regulates the number of epidermal growth factor receptors on human breast cancer cells. Thus, 1,25-$(OH)_2D_3$ has been shown to affect two steps in the regulation of cellular replication and, by analogy to similar changes in cell cycle progression caused by serum depletion, may affect growth factor synthesis as well (Fig. 4).

The inhibition of the growth of the colonic cancer cell (COLO 206F) xenografts is unequivocal with the 1,25-$(OH)_2D_3$-treated xenografts being less than 1% of the relative volume of the control xenografts, consistent with the presence of receptor in these cell line-derived solid tumor xenografts (Fig. 3). The rapid recovery of growth of the 1,25-$(OH)_2D_3$-treated xenografts when the hormone is withdrawn indicates that treatment must be continuous for effective control of tumor growth. Interestingly, the inhibitory effect on replication has also been shown to require continuous exposure to 1,25-$(OH)_2D_3$ in recent studies in leukemic cells *in vitro* (34). Moreover, this transient effect is also seen with antiestrogens, which do have an important role in the clinical management of breast cancer.

The marked inhibition of growth of 1,25-$(OH)_2D_3$ receptor-positive human xenografts as well as the data on cell cycle kinetic changes and down-regulation of EGF receptors indicate the significance of 1,25-$(OH)_2D_3$ in the regulation of cellular replication. This role, which is particularly relevant to the control of human cancer, clearly deserves further study. The development of less hypercalcemic analogues of 1,25-$(OH)_2D_3$ with enhanced and perhaps longer lasting antiproliferative activity (35) is particularly interesting

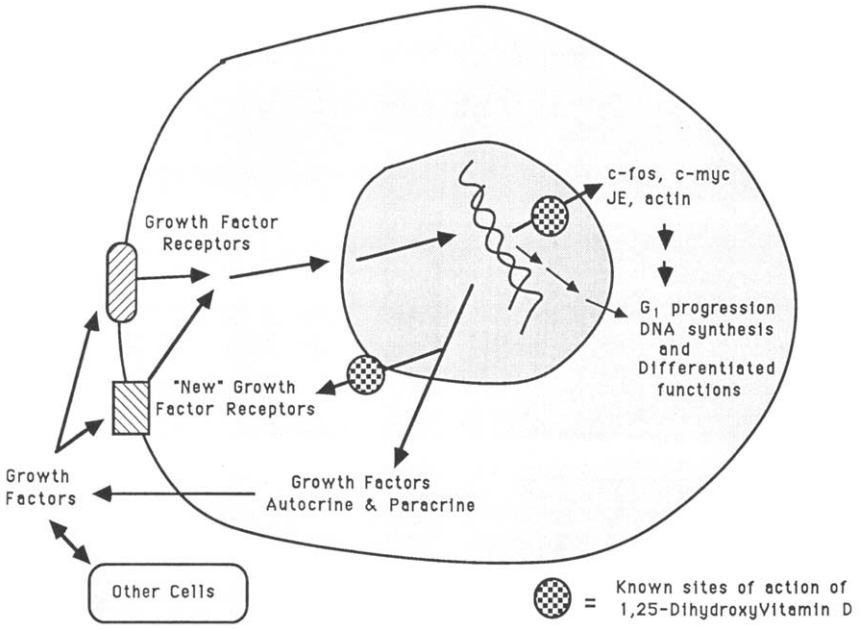

Figure 4. Diagram of some of the recognized points of regulation of cellular replication indicating those at which 1,25-$(OH)_2D_3$ effects have been demonstrated.

in this regard. Furthermore, investigation of the mechanism(s) of action of 1,25-$(OH)_2D_3$ in these cancer cell lines may provide further insight into those mechanisms which are disturbed in the development and progression of human cancer.

This study was supported by grants from the National Health and Medical Research Council of Australia, the New South Wales State Cancer Council, the Anti-Cancer Council of Victoria and Hoffmann-La Roche.

We thank Jean-Charles Fragonas, Mary Lynne McMenemy, Grace Pang, and Narelle Harley for technical assistance and Elizabeth Musgrove for valuable discussion.

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