Suppression of *in Vivo* Growth of Human Cancer Solid Tumor Xenografts by 1,25-Dihydroxyvitamin D₃¹

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ABSTRACT

It has been demonstrated previously that several human cancer cell lines possess specific, high affinity receptors for 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃, calcitriol] and that 1,25-(OH)₂D₃ and certain of its metabolites inhibit the growth *in vitro* of several human breast cancer and malignant melanoma cell lines, *i.e.*, analogous to the effect of estrogens on breast cancer. Furthermore, it has been shown that 1,25-(OH)₂D₃ and one of its synthetic analogues prolonged the survival in immune-suppressed mice. However, until now no growth-inhibitory effect of 1,25-(OH)₂D₃ has been demonstrated *in vivo* for human cancer cells or for solid cancers. This paper describes the suppression by 1,25-(OH)₂D₃ of the growth of human cancer cell-derived xenografts in immune-suppressed mice. However, the 24-hydroxylated metabolite and the 24-difluorinated analogue of 1,25-(OH)₂D₃, both of which are active *in vitro*, were ineffective in this xenograft model system. The suppression by 1,25-(OH)₂D₃, which was achieved without significant toxicity, was observed with xenografts derived from two 1,25-(OH)₂D₃ receptor-positive cell lines (COLO 206F, derived from a colon cancer, and COLO 239F from a malignant melanoma) but not in those from a receptor-negative line (RPMI 7932, also derived from a malignant melanoma). These studies demonstrate that pharmacological doses of 1,25-(OH)₂D₃ can be tolerated in the presence of a low calcium diet and that these doses can suppress the growth of human solid xenograft tumors *in vivo*. This is the first report of 1,25-(OH)₂D₃ growth suppression of solid tumors derived from human cancer cells in an *in vivo* model system, and it supports the hypothesized growth-inhibitory effect *in vivo* could have a wide range of potential applications in therapy of human malignancy. It has still to be demonstrated that the active metabolites of vitamin D₃ are able to affect the growth of human cancers *in vivo* or that these compounds can affect the growth of solid tumors as well as hematological malignancy. An obvious limitation to such use of the active hormonal form of vitamin D₃ is the potential hypercalcemic side effect of the high doses which would appear to be required. Thus two questions were approached in the present study. Do the active vitamin D₃ metabolites control the growth of human solid tumors *in vivo*? Can certain of these compounds be administered in effective doses without unacceptable toxicity?

MATERIALS AND METHODS

Toxicity of vitamin D₃ metabolites and analogues was studied initially in normal mice weighing 20 to 25 g (comparable to immunosuppressed mice, see below) maintained on a low calcium (0.1%) diet based on soybean flour. Groups of three to six mice were treated every other day with i.p. injections of 0.05 ml of corn oil vehicle (control), 1,25-(OH)₂D₃ (0.1 µg), 24,24-F₂-1,25-(OH)₂D₃ (0.02 µg), or 1,24,25-(OH)₃D₃ (2 µg) for 45 days. Vitamin D₃ metabolites and analogues were supplied by Dr. M. Uskokovic, Hoffmann-La Roche, Nutley, NJ. Serum calcium and body weight, as an index of general well-being, were determined at intervals during the study, throughout which the animals remained apparently well. Higher doses of each metabolite were tested at times, but these resulted in significant hypercalcemia even in animals on a low calcium diet (see below). In some studies mice, maintained on normal laboratory chow, were given dexamethasone (2 µg) i.p. every second day to suppress intestinal calcium absorption. Although this was effective in controlling the hypercalcemia, the animals were not in good health and were unable to maintain weight. Mice were thereafter maintained on the low (0.1%) calcium diet without any glucocorticoid therapy. At termination of the study the animals were killed under anesthesia, and blood was collected for estimating hematological parameters. One kidney was removed from each animal, cleaned of perirenal fat, and desiccated to obtain dry weight. It was then dissolved with concentrated nitric acid, and the calcium content was determined by atomic absorption spectrometry. The entire lower limb of each animal was removed, desiccated, and then subjected to "cleaning" by the beetle, *Dermestes maculatus*. The combined dry weight of the femur, tibia, and fibula was then determined. Serum calcium and lower limb bone weight were measured for evidence of effects on calcium and bone homeostasis, serum creatinine and renal calcium for evidence of nephrotoxicity, and full blood counts for evidence of hematological disturbance.

Immunosuppression of male and female CBA/Lac mice was achieved as previously described (12–14). Briefly mice were thymectomized at 16 to 18 days of age. After a further 18 to 21 days an i.p. injection of 1-β-D-α-arabinofuranosylcytosine (200 mg/kg) (Cytoxan; Upjohn Company, Kalamazoo, MI) was given and followed 48 h later by 8.5 G of whole-body irradiation using a 137Cs Gamagamma irradiation device (Atomic Energy of Canada, Ltd., Ottawa, Canada). Animals were used as xenograft hosts within 4 wk of irradiation (12–14). Cultures of human cancer cell lines were grown to near confluence in monolayer culture in Roswell Park Memorial Institute Medium 1640 (RPMI 1640) containing 5% fetal calf serum. They were harvested mechanically using a rubber scraper, washed in serum-free medium, resuspended, and...
injected s.c. (10⁵ to 10⁶ cells/site) into each flank of the immunosuppressed mice. Successful "takes" and the production of solid tumors were achieved with three cell lines. These cell lines, two malignant melanoma cell lines (COLO 239F and RPMI 7932) and one colorectal cancer cell line (COLO 206F), had been obtained initially from Dr. George Moore, Denver, CO. Xenografts obtained in the first group of immunosuppressed mice were removed, minced, and passaged in further immunosuppressed mice to establish stable xenografted tumor lines for the subsequent growth studies.

After xenografts had grown to a diameter of 8 to 10 mm, the greatest and least diameters were measured every second or third day using Vernier calipers, and tumor volumes were estimated using the formula 0.5πd², where d is the mean diameter. The volume of each tumor on the day of the commencement of assessment (V₀) was divided by the volume of the same tumor on the day of the commencement of assessment (Vₐ) to obtain the relative tumor volume (Vₐ/V₀). Graphs of log₁₀ Vₐ/V₀ versus time were constructed for control and treated xenografts and analyzed as linear regressions using the method of least squares. Regression coefficients were compared by analysis of variance. Equal numbers of male and female mice were randomly assigned to experimental and control groups with each group containing 10 to 14 xenografts. Control xenograft-bearing animals received either no treatment, low calcium diet and the toxicity studies in non-tumor-bearing mice. These doses had been selected on the basis of in vitro sensitivity (8, 9).

RESULTS

Moderate hypercalcemia occurred at Day 26 in all treated groups, but was not present at 45 days (Table 1). Control and 1,25-(OH)₂D₃-treated mice tended to gain weight over the 45-day period, while the mice treated with the other two vitamin D₃ compounds tended to lose weight; these differences were not statistically significant. Although lower limb bone weight tended to decline with treatment, this was not significant even with the use of a one-tailed t test. There were no significant changes in renal dry weight, calcium content, or in any of the blood parameters. However, renal calcium tended to rise, while dry weight of the lower limb bones tended to fall in those animals treated for 45 days with these compounds. The trihydroxylated compound, 1,24,25-trihydroxyvitamin D₃, had no significant effect on any of these parameters. The compounds tested did not have significant effects on the hematological parameters. There was no subjective evidence of clinical illness in any of the animal groups, and all animals survived the treatment period.

In an earlier experiment serum creatinine did not increase over 3-wk treatment with either 1,25-(OH)₂D₃ at 0.2 μg or 24,24-F₂-1,25-(OH)₂D₃ at 0.04 μg every other day. After 4 wk, however, there was a rise in creatinine in both treated and control animals: control, 0.06 ± 0.01; 1,25-(OH)₂D₃, 0.11 ± 0.02; and 24,24-F₂-1,25-(OH)₂D₃, 0.07 ± 0.02 mmol/liter. The more marked rise in creatinine in the treated animals was associated with significant rises in serum calcium: control, 2.5 ± 0.3; 1,25-(OH)₂D₃, 2.7 ± 0.3; and 24,24-F₂-1,25-(OH)₂D₃, 3.2 ± 0.1 mmol/liter. In other studies the animals treated even with the lower doses of the active compounds developed hypercalcemia within a few days of being transferred from the low calcium diet to a standard laboratory chow (data not shown). These studies therefore established the maximum tolerable doses of these vitamin D₃ compounds in terms of animal well-being and hypercalcemic toxicity. They led to the selection of the doses used in the xenograft growth studies described.

For the COLO 206F colon carcinoma xenografts, volume doubling time in control mice was 7 days (Fig. 1). The growth of these xenografts was markedly inhibited by treatment with 1,25-(OH)₂D₃ over the entire treatment period: Days 0 to 4, P < 0.05; Days 0 to 10, P < 0.005; and Days 0 to 25, P < 0.001. In fact, at the end of the study period, the relative tumor volume in the treated mice was less than 0.5% of that in the control
The melanoma line RPMI 7932 xenografts had a volume doubling time of 8 days (Fig. 2). The growth of these xenografts was unaffected by any of the treatments administered (Fig. 2). The other malignant melanoma xenograft line COLO239F also had a volume doubling time of 8 days (Fig. 3). However, while the control and treated tumors of these xenografts initially grew at similar rates, from Day 12 onwards the treated group grew significantly more slowly ($P < 0.05$) than control animals (Fig. 3). Over the entire 30 days of the experiment, the volume doubling time for control xenografts was 8 days compared to 27 days for treated animals ($P < 0.01$). Although the receptor level in the COLO 239F cells was too low to allow Scatchard analysis, single saturating dose analysis indicated a receptor concentration of $4.0 \pm 1.6$ (SE) fmol/mg of protein, $n = 3$. Receptor could not be demonstrated in RPMI 7932 cells.

**DISCUSSION**

*In vitro* addition of the active vitamin D$_3$ metabolites every other day is as effective, if not more effective, than daily addition in inhibiting human breast cancer cell (T47D) replication. This is despite the fact that, in these cells *in vitro*, there is rapid induction of 1,25-(OH)$_2$D$_3$ metabolism upon exposure of the cells to the hormone, such that virtually all the hormone has been metabolized within 8 h of its addition (17). Treatment every other day with the vitamin D$_3$ compounds was elected on the basis of these studies and also for simplicity of the treatment protocol.

It was surprising that the mice tolerated the high doses of the various vitamin D$_3$ compounds without apparent side effects. The efficacy of the low calcium diet in controlling the hypercalcemia indicated that intestinal calcium absorption was the major mechanism of this side effect. The rapid onset of hypercalcemia in treated mice transferred to normal laboratory chow supports this view. The small changes in renal ash calcium and lower limb weight in treated mice maintained on the low calcium diet, although not statistically significant, do suggest minor disturbance of bone and calcium metabolism. The transient rise in serum calcium at Day 26 (Table 1) is of some interest. It suggests the development of tolerance, but the reason for such apparent tolerance is not known. It may be mediated by mechanisms similar to the marked induction of metabolism of the hormone (17) and the “down-regulation” of functional receptor (18) observed in hormone-treated cancer cells.

In view of the recent findings of 1,25-(OH)$_2$D$_3$ receptors in cells of the immune system and the functional effects of 1,25-(OH)$_2$D$_3$ in both malignant and normal immune cells, it was particularly important to seek evidence of abnormalities of blood parameters. However, as shown in Table 1 there were certainly no gross changes in any of the hematological parameters nor were there any problems with infections or the like in the treated animals (data not shown).

The inhibition of the growth of the colonic cancer cell (COLO 206F) xenografts is unequivocal with the 1,25-(OH)$_2$D$_3$-treated xenografts being less than 1% of the relative volume of the control xenografts (Fig. 1). Although 1,25-(OH)$_2$D$_3$ did not cause regression of either of the malignant melanoma xenografts, it clearly suppressed the growth of the COLO 239F xenografts (Figs. 2 and 3). The COLO 206F cell line possesses the 1,25-(OH)$_2$D$_3$ receptor in moderate amounts (12 to 13 fmol/mg of protein) by a single saturating dose or Scatchard analysis (4). The COLO 239F cell line had not been found previously...
to have 1,25-(OH)_{2}D_{3} receptors (4); however, reexamination of the cell line currently in use with improved methods (16) showed a low level of receptor (4 fmol/mg of protein). It is of interest to note that the unresponsive line RPMI 7932 lacks 1,25-(OH)_{2}D_{3} receptors {	extit{in vitro}}; hence, the response to the hormone is consistent with the presence and level of receptor in these cell line-derived solid tumor xenografts.

The failure of the difluorinated analogue to inhibit the growth of the colonic cancer (COLO 206F) xenograft, in contrast to the activity of 1,25-(OH)_{2}D_{3}, is difficult to explain (Fig. 1). Although the difluorinated compound was administered at one-fifth the dose of 1,25-(OH)_{2}D_{3}, in {	extit{in vivo}} studies in malignant melanoma and breast cancer cells, it is a 5- to 10-fold more potent inhibitor of both anchorage-dependent (monolayer) and anchorage-independent (soft agar) replication (15). Similarly the trihydroxylated metabolite, administered at a 20-fold higher dose than that of 1,25-(OH)_{2}D_{3}, was inactive in these {	extit{in vivo}} studies, yet {	extit{in vitro}} it has about one-tenth the activity of the native hormone (9). It remains to be established whether these differences reflect differences in the metabolic capabilities of the different cell lines studied or whether the intact animal has metabolic activities which allow it to dispose of these modified compounds.

Some insight into the mechanism of the effects of 1,25-(OH)_{2}D_{3} on these solid tumors may be found in studies of hemopoietic cells. Several normal and malignant cells of these lineages possess 1,25-(OH)_{2}D_{3} receptor and respond to the hormone with inhibition of replication and stimulation of differentiation (19–24). In one malignant leukemic cell line (HL-60) the differentiating action is associated with a marked inhibition of the expression of the cellular oncogene c-myc (25). These studies suggest that 1,25-(OH)_{2}D_{3} has profound effects in controlling normal hemopoietic cell replication and differentiation and has a significant immunoregulatory role. By analogy with these reports and our previous studies in human breast cancer and malignant melanoma cells {	extit{in vitro}}, it would appear that 1,25-(OH)_{2}D_{3} has a range of effects on cellular replication and differentiation which are quite distinct from its well-recognized effects on calcium and bone homeostasis. The exact mechanisms by which 1,25-dihydroxyvitamin D3 suppresses growth in these human solid tumor xenografts are not yet clear. It would appear from previous {	extit{in vitro}} studies (4, 5, 7–9) that it acts directly on the tumor cells via intracellular receptors. There is also some evidence for promotion of cell differentiation (19–24). These mechanisms are not mutually exclusive, and it may be that they are mediated through inhibition of certain tumor growth factors, oncogenes, etc. (25). The fact that there were no deleterious effects of the high dose 1,25-(OH)_{2}D_{3} therapy on the hematological parameters studied in these animals supports the concept that 1,25-(OH)_{2}D_{3} specifically stimulates differentiation and thus has little or no additional effect on cells, which are already committed to a normal differentiation pathway. It is possible that 1,25-(OH)_{2}D_{3} may not be affecting the xenografts directly but instead may be acting to modify some host factor, e.g., the mouse stromal cells. However, if this were the case, all three xenografts or at least both malignant melanomas could be expected to respond equally. This possibility seems most unlikely in view of the specificity of the response. The correlation between the response and the 1,25-(OH)_{2}D_{3} receptor level in the three xenografts also supports a specific effect mediated in some way through the 1,25-(OH)_{2}D_{3} receptor.

The dose of 1,25-(OH)_{2}D_{3} is very high, representing a dose in a 70-kg person of 350 μg on a surface area basis, at least an order of magnitude greater than the normal daily production of this hormone in humans of about 1 μg/day. The dose is also an order of magnitude greater than that used by Honma \textit{et al.} (11) in their leukemic mouse study in which dietary calcium was presumably normal. The use of 1,25-(OH)_{2}D_{3} at equivalent doses in humans would be expected as in these studies to necessitate a low calcium diet. However, although there is some evidence of toxicity in our studies, this must be considered in the light of the underlying disease process for which the therapy could be proposed, \textit{i.e.}, metastatic malignancy. The marked inhibition of growth of two of these 1,25-dihydroxyvitamin D3 receptor human xenografts with relatively low toxicity supports the need for further studies, particularly with regard to toxicity, as steps towards the (eventual) trial of this hormone in human malignancy.

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