Inhibition of Human Cancer Cell Growth by 1,25-Dihydroxyvitamin D₃ Metabolites

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INTRODUCTION

Since the description of specific high-affinity 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃] receptors, which can undergo hormone-dependent activation and nuclear localization, have been demonstrated in a wide variety of established human cancer cell lines and surgically obtained human cancer tissues. 1,25-(OH)₂D₃ has been reported by some workers to stimulate cancer cell replication at low "physiological" concentrations and by ourselves and others to inhibit at higher concentrations. We report here that 1,25-(OH)₂D₃ had a biphasic effect on the replication of two distinct human cancer cell lines, i.e., the breast cancer T-47D and the malignant melanoma MM96, an effect analogous to that of estrogens on the breast cancer cell line MCF-7. These inhibitory effects were accompanied by marked morphological changes. Furthermore, two known metabolites of 1,25-(OH)₂D₃, i.e., 1,24,25-trihydroxyvitamin D₃ and 1,25,26,27-tetrahydroxyvitamin D₄, which compete for binding to the 1,25-(OH)₂D₃ receptor, did not stimulate but were almost equipotent with 1,25-(OH)₂D₃ in inhibiting the replication of both cell lines. The stimulatory but not the inhibitory effect of 1,25-(OH)₂D₃ was abolished by cortisone. These 1,25-dihydroxyvitamin D₃ metabolites show promise for the inhibition of cancer growth, analogous to the effect of estrogens and antiestrogens in breast cancer but with potential application in a much wider range of human cancers.

MATERIALS AND METHODS

Cells were trypsinized with 0.0125% trypsin in 0.02% disodium EDTA (Versene; CSL, Parkville, Australia), rinsed with phosphate-buffered saline, and subcultured into 6- or 12-well multiwell plates at densities of about 10⁴ cells/sq cm with a plating efficiency of 30 to 60%. After an initial overnight incubation (18 to 24 hr) in 10% FCS in RPMI 1640 (Flow Laboratories Australia, Ltd., Melbourne, Australia), the cells were changed to 1 to 2% untreated FCS or 5% charcoal-treated FCS (15) in RPMI 1640 supplemented with 0.1 μg/ml insulin (Novo Laboratories Pty. Ltd., Parramatta, Australia). Vitamin D₃ metabolites were the generous gift of Dr. M. Uskokovic (Hoffmann-La Roche Inc., Nutley, N. J.). Cortisone was purchased from the Sigma Chemical Company, St. Louis, Mo., and estradiol from BDH Biochemicals, Poole, United Kingdom. All steroids were added to the culture media in ethanol, the final concentration of which was 0.02% in control and 5% charcoal-treated media. Cells were harvested by trypsinization at appropriate times up to confluence. Cell numbers were determined by Coulter Counter and by hemocytometer. Cell protein (16) and DMA (14) content per well were determined on portions of the harvested cells. At times, specific binding of [³H]-1,25-(OH)₂D₃ was analyzed in intact T-47D cells as described previously (19). In brief, confluent T-47D cells in multiwell dishes were incubated in RPMI 1640 containing 0.01% bovine serum albumin with 0.5 μM 1,25-(OH)₂[³H]-vitamin D₃ (80 to 163 Ci/mmol; Amersham, Bucks, United Kingdom) alone or with various concentrations of vitamin D₃ metabolites. After incubation at 37°C for 6 hr, the medium was removed, and the cells were rinsed once with phosphate-buffered saline (Oxoid, Ltd., United Kingdom) containing 0.1% bovine serum albumin and twice with phosphate-buffered saline alone. Cells were solubilized with NaOH, neutralized with acetic acid, and counted with Instagel scintillant (Packard Instrument Co., Inc., Downers Grove, Ill.) in a Packard Tri-Carb scintillation counter.

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1 This research was supported by the National Health and Medical Research Council and the Victorian Anti-Cancer Council.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃; 1,25-DR, 1,25-dihydroxyvitamin D₃ receptor; FCS, fetal calf serum; RPMI 1640, Roswell Park Memorial Institute Medium 1640; 25-OH D₃, 25-hydroxyvitamin D₃; 24,25-(OH)₂D₃, 24,25-dihydroxyvitamin D₃; 1,24,25-(OH)₃D₃, 1,24,25-trihydroxyvitamin D₃; 1,25,26,27-tetrahydroxyvitamin D₄.

ABSTRACT

Specific high-affinity 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃] receptors, which can undergo hormone-dependent activation and nuclear localization, have been demonstrated in a wide variety of established human cancer cell lines and surgically obtained human cancer tissues. 1,25-(OH)₂D₃ has been reported by some workers to stimulate cancer cell replication at low "physiological" concentrations and by ourselves and others to inhibit at higher concentrations. We report here that 1,25-(OH)₂D₃ had a biphasic effect on the replication of two distinct human cancer cell lines, i.e., the breast cancer T-47D and the malignant melanoma MM96, an effect analogous to that of estrogens on the breast cancer cell line MCF-7. These inhibitory effects were accompanied by marked morphological changes. Furthermore, two known metabolites of 1,25-(OH)₂D₃, i.e., 1,24,25-trihydroxyvitamin D₃ and 1,25,26,27-tetrahydroxyvitamin D₄, which compete for binding to the 1,25-(OH)₂D₃ receptor, did not stimulate but were almost equipotent with 1,25-(OH)₂D₃ in inhibiting the replication of both cell lines. The stimulatory but not the inhibitory effect of 1,25-(OH)₂D₃ was abolished by cortisone. These 1,25-dihydroxyvitamin D₃ metabolites show promise for the inhibition of cancer growth, analogous to the effect of estrogens and antiestrogens in breast cancer but with potential application in a much wider range of human cancers.
RESULTS

In the presence of 1 to 2% untreated PCS, 1,25-(OH)2D3 inhibited replication of T-47D and MM96 cells by 30 to 50% at 10^-8 M. There was no stimulation of replication at concentrations of 1,25-(OH)2D3 from 10^-10 to 10^-8 M (Chart 1A). Several other vitamin D3 metabolites, including 25-OH D3, 24,25-(OH)2D3, and 1,24,25-(OH)3D3, had no significant effect on replication. Another metabolite, 1,25,26-(OH)3D3, inhibited replication at the same concentrations at which 1,25-(OH)2D3 was inhibitory. On the other hand, when the cells were cultured in the presence of 5% charcoal-treated PCS, 1,25-(OH)2D3 stimulated replication by up to 50%, with a maximum effect at between 1 and 5 x 10^-11 M (Chart 2), but was still inhibitory at higher concentrations (10^-8 M) (Fig. 1B; Table 1). The doubling times of the T-47D cells were indistinguishable in the 2 PCS media, approximately 62 hr in each case. In the charcoal-treated FCS medium and at the same concentration as 1,25-(OH)2D3, 1,24,25-(OH)3D3 and 1,25,26-(OH)3D3 markedly inhibited replication of either cell line. Both 25-OH D3 and 24,25-(OH)2D3 inhibited replication to a small extent at 10^-7 M (data not shown). Apart from 1,25-(OH)2D3, none of the vitamin D3 metabolites tested in several experiments stimulated the replication of the cancer cells (Chart 1B; Table 1). The various changes in cell number were paralleled by those in DNA both qualitatively and quantitatively; changes in protein content per well were similar qualitatively but were less marked. It is of interest that the stimulatory effect of 1,25-(OH)2D3 on T-47D replication could be completely inhibited by the presence of 10^-6 M (Chart 1). T-47D cells were subcultured in RPM1 1640 in the presence of various vitamin D3 metabolites at the concentrations indicated (E, 10^-10 M; F, 10^-8 M; G, 10^-6 M). Incubations were continued for 9 days in the presence of 1% untreated FCS (A) or for 8 days in the presence of 5% charcoal-treated FCS (B). Cell numbers are expressed as percentages of ethanol-treated controls, which were 2.33 x 10^6 cells/well (A) and 0.36 x 10^6 cells/well (B), respectively. Significances of differences from control: * p < 0.05; ** p < 0.01; *** p < 0.001. Bars, S.E. Stimulation of replication was seen only with low concentrations of 1,25-(OH)2D3 and in the presence of charcoal-treated FCS. Inhibition of replication was seen with high concentrations of 1,25-(OH)2D3 and 1,25,26-(OH)3D3 in either medium and with 1,24,25-(OH)3D3 in the charcoal-treated FCS medium.

Chart 1. T-47D cells were incubated in the presence of various D3 metabolites at the concentrations indicated (E, 10^-10 M; F, 10^-8 M; G, 10^-6 M). Incubations were continued for 9 days in the presence of 1% untreated FCS (A) or for 8 days in the presence of 5% charcoal-treated FCS (B). Cell numbers are expressed as percentages of ethanol-treated controls, which were 2.33 x 10^6 cells/well (A) and 0.36 x 10^6 cells/well (B), respectively. Significances of differences from control: * p < 0.05; ** p < 0.01; *** p < 0.001. Bars, S.E. Stimulation of replication was seen only with low concentrations of 1,25-(OH)2D3 and in the presence of charcoal-treated FCS. Inhibition of replication was seen with high concentrations of 1,25-(OH)2D3 and 1,25,26-(OH)3D3 in either medium and with 1,24,25-(OH)3D3 in the charcoal-treated FCS medium.

Chart 2. Cells were incubated in the presence of 1,25-(OH)2D3 at the concentrations indicated in media containing 5% charcoal-treated FCS. Cell numbers and DNA content per well was estimated after 13 days with breast cancer (T-47D) cells (A) and after 8 days with malignant melanoma (MM96) cells (B). Bars, S.E. In T-47D cells (A), stimulation of both cell number and DNA content was maximal at about 5 x 10^-11 M 1,25-(OH)2D3 and was significant (p < 0.05) with respect to control at all concentrations up to 5 x 10^-10 M. In MM96 cells (B), stimulation was maximal at about 10^-11 M 1,25-(OH)2D3 and was significant at all concentrations up to 2.5 x 10^-10 M.
Table 1
Effects of vitamin D3 metabolites on replication of malignant melanoma (MM96) cells

Cells were subcultured for 3 days in the presence of 5% charcoal-treated FCS with the various vitamin D3 metabolites at the concentrations indicated. Significant stimulation of replication is only seen with 1,25-(OH)2D3. Inhibition of replication by 1,25,26-(OH)3D3 and 1,24,25-(OH)2D3 is seen at concentrations similar to that required for inhibition by 1,25-(OH)2D3. 25-OH D3 and 24,25-(OH)2D3 inhibit also, but at 10-fold higher concentrations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell no. (× 10^5)/well</th>
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<tr>
<td>Control (ethanol vehicle)</td>
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<tr>
<td>1,25-(OH)2D3</td>
<td>184 ± 5²</td>
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<tr>
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<td>10⁻⁶ M</td>
<td>132 ± 2</td>
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<tr>
<td>10⁻¹⁰ M</td>
<td>153 ± 3</td>
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<tr>
<td>10⁻⁸ M</td>
<td>148 ± 1</td>
</tr>
<tr>
<td>10⁻⁶ M</td>
<td>139 ± 2²</td>
</tr>
<tr>
<td>1,24,25-(OH)2D3</td>
<td>168 ± 4</td>
</tr>
<tr>
<td>10⁻¹⁰ M</td>
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<td>10⁻⁸ M</td>
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<tr>
<td>10⁻⁶ M</td>
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</tr>
<tr>
<td>25-OH D3</td>
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<td>153 ± 3</td>
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<tr>
<td>10⁻⁸ M</td>
<td>151 ± 4</td>
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<tr>
<td>10⁻⁶ M</td>
<td>137 ± 2²</td>
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<tr>
<td>10⁻¹⁰ M</td>
<td>138 ± 23</td>
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<td>10⁻⁸ M</td>
<td>153 ± 3</td>
</tr>
<tr>
<td>10⁻⁶ M</td>
<td>130 ± 6</td>
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</table>

* Mean ± S.E.

a p < 0.001, relative to control.

b p < 0.001, relative to control.

c p < 0.05, relative to control.

d p < 0.005, relative to control.

m cortisone, which did not alter the inhibitory effect or have any effect of its own on replication (Chart 3). Cortisone at this concentration had a similar effect on the stimulatory and inhibitory effects of estradiol (data not shown). Also, the stimulatory effects of estrogens and 1,25-(OH)2D3 on replication are not additive, but their inhibitory effects are (data not shown). These effects of the vitamin D3 metabolites on the replication of the cancer cells were first seen after 5 or 6 days of incubation and persisted and increased over the rest of the incubation until the cells approached confluence (data not shown). The effects of the various metabolites on the inhibition of growth was qualitatively similar to their ability to compete with [³H]-1,25-(OH)2D3 for specific binding to the intact T-47D cells (Chart 4). The inhibitory effect of 1,25-(OH)2D3 on cellular growth was accompanied by a morphological change from plump cells with smooth outlines to contracted cells with prominent processes (Fig. 1). The stimulatory effect was not accompanied by any morphological changes. In preliminary studies in another 1,25-(OH)2D3 receptor-positive cancer cell line, the colonic line COLO 357, 1,25-(OH)2D3 had similar inhibitory effects on cell replication.

DISCUSSION

This paper clearly demonstrates the biphasic dose effect of 1,25-(OH)2D3 on the replication of two 1,25-DR-positive human cancer cell lines of quite different issue origin, namely, breast cancer and melanoma. It shows that the discrepancy between previously reported stimulation and inhibition by the same concentration of the hormone in different laboratories is due to the culture medium used. The lack of stimulation by 1,25-(OH)2D3 in the medium containing untreated FCS could be due to a masking effect of endogenous hormone, as has been proposed for the absence of the estrogen stimulation of MCF-7 cells in media containing untreated FCS (15). It seems more likely that the stimulatory effect of 1,25-(OH)2D3 on cancer cell replication depends upon complex factors in the culture medium, including the concentrations of various hormones, fatty acids, etc., which are altered by charcoal treatment. Since the stimulatory effects of
estrogen and 1,25-(OH)₂D₃ are not additive and since the stimulatory effect of 1,25-(OH)₂D₃ is blocked by glucocorticoids, it is possible that concentrations of these hormones in the untreated FCS could be masking the effect of added 1,25-(OH)₂D₃. Indeed, residual levels of 1,25-(OH)₂D₃ in the FCS could be masking the effect of added hormone. However, the reason for the discrepancy caused by the FCS used has not been specifically addressed in this paper or by other workers in the estrogen field.

The more important point of the present data, however, is the persistence and magnitude of the inhibitory effect of 1,25-(OH)₂D₃ on both cell lines irrespective of medium conditions. Since the 1,25-(OH)₂D₃ effect occurs at a much lower concentration in vitro than that of estradiol (10⁻⁶ and 10⁻⁸ M, respectively), these data strongly suggest that these effects could be exhibited in vivo at achievable concentrations. However, even these concentrations of 1,25-(OH)₂D₃ would cause marked bone resorption and hypercalcemia in vivo. For several reasons, then, the 2 other vitamin D₃ metabolites, 1,24,25-(OH)₃D₃ and 1,25,26-(OH)₂D₃, are particularly promising: (a) these metabolites did not appear to stimulate cell replication but were almost equipotent with 1,25-(OH)₂D₃ in inhibiting cancer cell replication in vitro; (b) these metabolites have considerably less biological activity in vivo [1 to 10% of that of 1,25-(OH)₂D₃], particularly with respect to bone mineral mobilization (1, 21). It is possible, therefore, that these metabolites or even 25-OH D₃ or 24,25-(OH)₂D₃ could be used at tumor-inhibitory concentrations in vivo without producing unacceptable bone resorption and hypercalcemia; (c) since glucocorticoid does not block the inhibitory effect of 1,25-(OH)₂D₃ in vitro, the possibility of using glucocorticoids in vivo to help control hypercalcemia deserves investigation.

It is appropriate to consider the relationship between the level of 1,25-(OH)₂D₃ (10⁻⁸ M) added, which clearly causes inhibition, and the apparent Kᵣ of 0.7 x 10⁻¹⁰ M (19) of the 1,25-DR in the intact T-47D cells. Both cell types actively metabolize [³H]-1,25-(OH)₂D₃ to more polar compounds. In fact, under comparable conditions, approximately 80% of added [³H]-1,25-(OH)₂D₃ (8 x 10⁻⁸ M) was metabolized in 13 hr. Hence, the mean level of hormone over the 2-day period between medium changes will be, clearly, well below the initial added concentration. Furthermore, the relative inhibitory potencies of the various metabolites [1,25-(OH)₂D₃ > 1,24,25-(OH)₃D₃ > 1,25,26-(OH)₂D₃ > 25-OH D₃ > 24,25-(OH)₂D₃] closely parallels their ability to bind to the receptor in the intact cell (Chart 4). These data support the concept that the inhibitory effect on cell replication of these vitamin D₃ metabolites may be mediated through the 1,25-DR.

On the other hand, the role of the receptor in the stimulation is much less clear. The other vitamin D₃ metabolites, which do compete for receptor binding, do not stimulate replication significantly. Hence, it is unlikely that this effect is being mediated through the receptor as currently understood. It is possible that 1,25-(OH)₂D₃ exerts some allosteric effect on the receptor, which determines the effect of its interaction with the nucleus and which cannot be induced by the other metabolites. This area deserves further investigation.

In summary, then, the data presented in this report indicate that vitamin D₃ metabolites or analogues could be effective antitumor agents in vivo. Since various reports (2–12, 17, 18, 20) demonstrate that 1,25-(OH)₂D₃ receptor positivity is a very common finding in human cancer cell lines, it can be seen that these vitamin D₃ metabolites have a much wider potential for application in human cancer than do the antiestrogens. The extension of these studies to in vivo models and the testing of other vitamin D₃ metabolites and analogues for antitumour activity are essential.

REFERENCES

Fig. 1. T-47D cells were incubated in RPMI 1640 containing 5% charcoal-treated FCS for 8 days in the presence (A) or absence (B) of $10^{-8}$ M 1,25-(OH)$_2$D$_3$. Control cells (B) are plump, with few processes, while 1,25-(OH)$_2$D$_3$-treated cells (A) are contracted, with prominent cellular processes. Phase contrast, ×130.
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