Prevention of Preneoplastic Mammary Lesion Development by a Novel Vitamin D Analogue, 1α-Hydroxyvitamin D₅

Rajendra G. Mehta, Robert M. Moriarty, Rajeshwari R. Mehta, Raju Penmasta, Gianluca Lazzaro, Andreas Constantinou, Liang Guo*

Background: The form of vitamin D (vitamin D₃) in fortified milk and the provitamin D produced by the body undergo metabolic activation to a biologically active form, 1α,25-dihydroxyvitamin D₃ [1α,25(OH)₂D₃]. This compound can induce cell differentiation and can prevent proliferation of cancer cells. However, because 1α,25(OH)₂D₃ is hypercalcemic (effective in increasing serum calcium level), it is not suitable for use in cancer prevention or cancer therapy trials. Purpose: We synthesized a vitamin D₃ series analogue, 1α-hydroxy-24-ethyl-cholecalciferol, or 1α-hydroxyvitamin D₅ [1α(OH)D₅], and evaluated its chemopreventive activity in carcinogen-treated mammary glands in organ culture experiments. Methods: The analogue 1α(OH)D₅ was synthesized from sitosterol acetate and was characterized by nuclear magnetic resonance. Its purity was evaluated by high-pressure liquid chromatography. The calcemic activities of vitamin D₃ and D₅ analogues were determined in vitamin-D-deficient Sprague-Dawley rats. Mammary glands of BALB/c mice were placed in organ culture and treated with the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) to induce preneoplastic lesions. Vitamin D analogues were added to the culture medium at four different concentrations, and formation of mammary lesions was evaluated. The effects of 1α(OH)D₃ and 1α,25(OH)₂D₃ on the expression of vitamin D receptors (VDRs) and transforming growth factor-β1 (TGF-β1) were studied by immunohistochemistry. Statistical significance was determined by the chi-squared test. All reported P values were two-sided. Results: 1α,25(OH)₂D₃ was fourfold more calcemic than 1α(OH)D₅ at a dose of 0.042 μg/kg per day in rats. Both 1α,25(OH)₂D₃ and 1α(OH)D₅ inhibited the development of DMBA-induced preneoplastic lesions in mouse mammary glands compared with untreated glands. The effect of the vitamin D₅ analogue was observed at a much lower concentration (0.01 μM). Treatment with 1α(OH)D₅ resulted in a dose-related (0.01-10.0 μM) inhibition without any toxicity, whereas the vitamin D₃ analogue was highly potent but toxic at concentrations of 1.0 μM or higher. Normal mouse mammary glands poorly express VDR and TGF-β1; incubation with 1α(OH)D₅ or 1α,25(OH)₂D₃ dramatically induced their expression. Conclusions: This is the first report showing the possibility of chemoprevention by a vitamin D₅ series compound. We conclude that 1α(OH)D₅ is less calcemic than 1α,25(OH)₂D₃. It is nontoxic at a wide range of concentrations, but it is potent in inhibiting the development of preneoplastic lesions in mammary glands in organ culture. In addition, we show for the first time the induction of TGF-β1 in normal mammary tissues by a chemopreventive agent. Implications: 1α(OH)D₅ is a good candidate for in vivo chemoprevention studies. It may mediate its action by inducing expression of VDR and of TGF-β1, as is seen in other systems. [J Natl Cancer Inst 1997;89:212-8]

Vitamin D is a secosteroid and is classified as a hormone within a steroid hormone family (1,2). It has been separated on the basis of its chemical structure into different series, e.g., D₂, D₃, D₄, D₅, and D₆. To date, attention has been focused almost exclusively on the vitamin D₃ series of compounds. In its biologic form, vitamin D₃ is inactive unless it is metabolized to 1α,25-dihydroxyvitamin D₃ [1α,25(OH)₂D₃]. The inactive 24-hydroxy form of the hormone is excreted from the body (3,4). The active metabolite 1α,25(OH)₂D₃ has been shown to suppress the growth in vitro of many neoplastic cells, including breast cancer cells (5,6). In addition, treatment of colon cancer cells and leukemia cells with 1α,25(OH)₂D₃ results in a reduction in the growth rate of these cells (7,8). One of the limiting factors in the successful use of vitamin D₃ in cancer prevention or cancer therapy is its calcemic activity (9). The concentrations of vitamin D₃ required to suppress growth of neoplastic cells cause hypercalcemia and death. Therefore, in recent years, numerous analogues of vitamin D have been synthesized that can reduce calcemic activity without compromising its antiproliferative activity (9,10). The differences in structures of these new compounds arise mostly from modifications in the C and D rings.

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See "Notes" following "References."
and side chain of the vitamin. No attempt, however, has been directed toward evaluating vitamin D compounds in other series. More work needs to be directed toward evaluating such compounds in other series.

The effectiveness of a variety of chemopreventive agents has been evaluated by organ culture of the mouse mammary gland (11-13). In organ culture, mammary glands from mice respond to a short stimulation with a carcinogen in the presence of appropriate hormones by developing preneoplastic lesions (14). When implanted in syngeneic hosts, mammary cells prepared from these lesions give rise to adenocarcinomas (15). Effective chemopreventive agents (e.g., certain retinoids, selenium, oltipraz, and limonene) inhibit the formation of these lesions. The relative activity of chemopreventive agents in vitro correlates well with their activity in vivo carcinogenesis experiments (13,15).

Using this model system, we have evaluated the efficacy of 1α-hydroxyvitamin D₃ [1α(OH)D₃] in preventing 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary lesion formation in a mouse mammary gland organ culture model.

The mechanism of the vitamin D action is not completely understood. Nuclear vitamin D receptor (VDR) protein binding to 1α,25(OH)₂D₃ has been identified and is shown to be present in a variety of tissues, including normal mammary glands and mammary tumors, as well as in breast cancer cells (17,18). In the cytosol of target organs or cells, [3H]1,25(OH)₂D₃ binds specifically to receptors with a dissociation constant (Kᵣ) ranging from 1 X 10⁻¹⁰ M to 6 X 10⁻¹⁰ M. An increased nuclear VDR concentration has been found to be associated with an enhanced expression of messenger RNA for vitamin D₃ receptors (19,20).

The VDR gene has been cloned, and the molecular structure of the receptor protein has been determined. The results have demonstrated that the VDR belongs to the steroid-, thyroid-, and retinoid-receptor superfamily (21). All of these receptors act as ligand-dependent transcription factors that bind to specific DNA sequences. Two classes of response elements have been identified that are activated either by VDR alone or by heterodimers of VDRs and retinoid X receptor (RXR) alpha (22,23).

In recent years, considerable attention has been given to the regulation of cell growth by autocrine antiproliferative factors [e.g., (24)]. Inhibition of cancer cell growth is often related to enhanced production of transforming growth factor-β (TGF-β) (25,26). TGF-β is further subclassified into the following three isoforms of polypeptides: TGF-β1, TGF-β2, and TGF-β3 (27).

These isoforms are present in mammalian cells, including breast cancer cells. The isoforms of TGF-β are regulated differentially by steroid and protein hormones. In one report (28), a hexaflouro analogue of vitamin D₃, 1α,25-dihydroxy-16-ene-23yne-26,27-hexafluorohexachlorofuran (Ro24-5531), induced expression in HL-60 human leukemia cells of TGF-β1 and its type 2 receptors. These results suggest a possible interaction between the function of VDR and TGF-β regulation. Induction of TGF-β, however, is often reported only in transformed cells. Although the growth-inhibitory role of TGF-β has been reported in the normal mammary gland (29), induction of TGF-β in response to chemopreventive agents in this tissue has not been reported previously.

In this study, we compare the chemopreventive action of vitamin D₃ with that of the active metabolite of vitamin D₃ and attempt to determine the possible mechanism of such action by studying the expression of VDRs and TGF-β in normal mammary epithelial cells.

Materials and Methods

Synthesis of 1α(OH)D₃. As a first step, β-sitosterol acetate was converted to 7-dehydro-β-sitosterol acetate by allelic bromination (using dibromomann and sodium bicarbonate in hexane and refluxing for 30 minutes) and dehydrobromination (using tetrabutylammonium fluoride, t-silidine, and tetaethylorthothymium and refluxing for 6 hours). 7-Dehydro-β-sitosterol was reduced to 7-dehydro-3β-sitosterol by refluxing with 6 hours with lithium aluminium hydride and tetraethylorthothymium. The reaction mixture was subjected to characterization and then to thermolysis (by ethanol reflux) to yield vitamin D₃. We converted vitamin D₃ to 1α(OH)D₃ by following the Paaren-Deluca hydroxylation sequence (30). 1α(OH)D₃ was crystalized, and the crystalline product was fully characterized by 1H nuclear magnetic resonance (NMR) at 400 Hz, mass spectrometry (chemical ionization), and infrared (IR) and ultraviolet spectrosocopy. The purity was determined by high-pressure liquid chromatography (HPLC). The following properties were recorded: melting point—150 °C-152 °C; IR (KBr) cm⁻¹—3349 (OH stretching) and 2955 (CH stretching); 1H NMR—86.35 (doublet [d], C-6H), 6.03 (d, C-7H), 5.33 (singlet [s], C-19H), 4.44 (multiple [m], C-11H), 4.24 (m, C-3H), and 0.55 (s, C-18H); mass spectrum (CI)—429 (M + 1, 72%), 111 (M + 1 — H₂O, 100%), and 393 (M + 1 — 2H₂O, 14%); and UV—λ max, 260 nm (molar extinction coefficient [ε] = 18,000).

HPLC analysis of vitamin D analogues. Vitamin D analogues were dissolved in acetone at a final concentration of 0.2 mg/mL. Aliquots (10 μL) were injected into a Supelco PKB-100 HPLC column at ambient temperature. The HPLC was carried out with the use of an Hitachi L-6000 pump, an L-4200 UV-VIS detector, and an AS-2000 autosampler (Hitachi Instruments, Inc., Naperville, IL). Vitamin D analogues were eluted with the mobile phase of acetone–methanol—water (52:30:18, vol/vol) with the flow rate at 1 mL/min, and the elution profile was monitored at 254 nm.

Measurement of calcemic activity. Three-week-old Sprague-Dawley male rats were obtained from the Holtzman Laboratory, Madison, WI. Up to three rats were housed together in a polycarbonate cage. The animal cages were kept under yellow light. The rats (eight to 10 per group per concentration of both vitamin D analogues used) were fed vitamin D-free diet containing 0.47 g/100 g calcium and 0.3 g/100 g phosphorus. After the rats had consumed this diet for 3 weeks, their plasma calcium levels were measured. Rats exhibiting plasma calcium levels of less than 6.0 mg/dL were considered to be vitamin D deficient. Such rats were administered appropriate vitamin D analogues intragastrically for 14 days. At the end of this period, the plasma calcium levels were again measured.

Induction of preneoplastic lesions in mammary glands and their prevention by vitamin D₃ and D analogues. Young, virgin BALB/c female mice, 3-4 weeks of age, were obtained from Charles River Laboratories, Wilmington, MA. The entire culture procedure was described in detail previously (12-14). Briefly, the mice were pretreated for 9 days with 17β-estradiol (1 μg in 0.1 mL saline per animal) and progesterone (1 mg in 0.1 mL saline per animal). They were then killed by cervical dislocation, and the thoracic pair of mammary glands was dissected out on silk ruffs and incubated for 10 days in Waymouth MB752 medium (Life Technologies, Inc., [GBCO BR], Gaithersburg, MD) containing the following growth-promoting hormones: insulin (5 μg/mL), prolactin (5 μg/mL), aldosterone (1 μg/mL), and hydrocortisone (1 μg/mL). The carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) at a dose of 2 μg/mL was added to the medium on day 3 for 24 hours to induce mammary lesions. The DMBA-containing medium was then removed, and the mammary glands were incubated for an additional 14 days with medium containing only insulin. This procedure allowed the normal glands to undergo structural rearrangement in which all the normal alveolar structures were disintegrated. However, the alveolar lesions in the carcinogen-treated glands behaved differently. They had acquired altered hormone responsiveness, and these structures did not regress. These structures were termed "mammary lesions." The vitamin D analogues (ranging in concentration from 0.01 μM to 10.0 μM) were included in the medium during the first 10 days of the in vitro culture to determine if they lowered the incidence of mammary lesion formation. Throughout the culture period, the glands were maintained at 37 °C in an environment of 95% air and 5% CO₂. The procedure is presented schematically in Fig. 1. At the end of the culture period, the glands were fixed in formalin, stained in alum-carmine solution, and evaluated for the presence or absence of mammary lesions. All hormones and chemicals were purchased from the Sigma Chemical Co., St. Louis, MO.
Immunohistochemistry of VDRs and TGF-β1. Normal mouse mammary glands were dissected and incubated with growth-promoting hormones either alone or in the presence of vitamin D analogues for only 3 days. In this experiment, the glands were not exposed to DMBA (see protocol described in the previous paragraph). The glands were fixed in buffered formalin, and 5-μm-thick sections were prepared for histopathologic evaluations. The sections were mounted on adhesive-coated slides (Superfrost; Fisher Scientific Co., Itasca, IL), dried at 60°C for 1 h, deparaffinized in xylene, dehydrated in a series of alcohol, and finally washed with phosphate-buffered saline (PBS). To block the nonspecific antibody reactions, we treated the tissue sections with 5% dried skim milk for 10 minutes and then incubated them with primary mouse antibody (either against VDR or against TGF-β1; both obtained from BioGenex Laboratories, San Ramon, CA) for 10 minutes; the remaining steps were followed according to the manufacturer-specified protocol; i.e., the reaction was stopped by rinsing the sections with PBS, which was followed by 10 minutes' incubation with peroxidase-conjugated streptavidin, three 10-minute rinses with PBS, and a 5-minute incubation in a substrate, 3,3′-diaminobenzidine tetrachloride. The tissues were counterstained with hematoxylin–eosin, dehydrated through graded series of alcohol and xylene, and finally mounted in Permount (Fisher Scientific Co.). Slides were evaluated for the presence or absence of the VDR or TGF-β1 and for the intensity of staining in the positively stained samples.

Statistical analysis. Statistical significance was determined by the chi-squared test. All reported P values were obtained from two-sided tests.

Results

We synthesized highly purified 1α(OH)D₃. The chemical structures of 1α,25(OH)₂D₃ and 1α(OH)D₃ are shown in Fig. 2. The major differences between these two molecules are the lack of hydroxylation at the C-25 position and the presence of ethyl group at the C-24 position in the newly synthesized vitamin D₃ analogue. The purity of these compounds was evaluated by HPLC analysis. The purity specifications are described in the “Materials and Methods” section. As shown in Fig. 2, 1α,25(OH)₂D₃ and 1α(OH)D₃ were eluted with retention times of approximately 5 and 34 minutes, respectively. Both of these vitamin D analogues exhibited about 98% purity. Stability studies have suggested that both can be stored in powder form for a year at −20°C, whereas in solution they are stable for 1 month at the same temperature. For all the experiments described in this article, stock solutions of vitamin D compounds were prepared fresh each time.

One of the primary reasons to synthesize new vitamin D agents is to prepare analogues that have reduced calcemic activity compared with that of 1α,25(OH)₂D₃, but without compromising the antiproliferative activity. We measured the calcemic activity of both of these vitamin D analogues. As shown in Table 1, the vehicle-treated control rats showed a plasma calcium concentration of 5.4 ± 0.3 mg/dL (mean ± standard deviation). When the rats were treated with vitamin D analogues at a dose of 0.042 μg/kg per day, the following plasma calcium concentrations were observed: 6.0 ± 0.63 mg/dL for 1α(OH)D₃-treated rats (11% increase over that of the vehicle-treated control group,
Table 1. Effects of vitamin D analogues on plasma calcium levels in vitamin D-deficient rats

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>No. of rats</th>
<th>Dose, µg/kg per day</th>
<th>Plasma calcium, mg/dL†</th>
<th>P‡</th>
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<tbody>
<tr>
<td>None</td>
<td>8</td>
<td>0</td>
<td>5.4 ± 0.28</td>
<td>.121</td>
</tr>
<tr>
<td>1α(OH)D₃</td>
<td>8</td>
<td>0.042</td>
<td>6.0 ± 0.63</td>
<td>.002</td>
</tr>
<tr>
<td>1α,25(OH)₂D₃</td>
<td>8</td>
<td>0.042</td>
<td>7.9 ± 1.5</td>
<td>.001</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.25</td>
<td>8.1 ± 1.2</td>
<td>.004</td>
</tr>
</tbody>
</table>

*1α(OH)D₃ = 1α-hydroxyvitamin D₃, 1α,25(OH)₂D₃ = 1α,25-dihydroxyvitamin D₃.
†Values = means ± standard deviation.
‡Two-sided test.

P = .121, not statistically significant when compared with that of the control group) and 8.1 ± 1.2 mg/dL for 1α,25(OH)₂D₃-treated rats (50% increase over that of the control group, P = .001, statistically significant difference when compared with that of the control group). At a higher concentration of vitamin D analogues (0.25 µg/kg per day), 1α(OH)D₃ treatment resulted in a plasma calcium concentration of 7.9 ± 1.5 mg/dL, compared with 10.1 ± 1.8 mg/dL for 1α,25(OH)₂D₃ treatment. Although both analogues at this concentration increased the plasma calcium levels in comparison with those in vehicle-treated control rats, these results showed that 1α(OH)D₃ had overall lower calcemic effects than 1α,25(OH)₂D₃. 1α,25(OH)₂D₃ treatment resulted in an 87% increase in the plasma calcium level in rats when compared with the vehicle-treated rats. On the other hand, 1α(OH)D₃, there was only a 50% increase in the plasma calcium concentration compared with that in the control animals. These results suggest that 1α(OH)D₃ is much less calcemic than 1α,25(OH)₂D₃.

To evaluate the efficacy of the newly synthesized vitamin D₃ analogue in preventing mammary lesion formation, we incubated 15 mammary glands per group (135 glands in total) from BALB/c mice with appropriate hormones and exposed the glands to DMBA on day 3 for 24 hours (see “Materials and Methods” section). The mammary glands were incubated for 10 days with the vitamin D analogues in concentrations ranging from 0.01 µM to 10.0 µM. The incidence of mammary lesions was calculated for each group and was reported as the ratio of the number of mammary glands showing lesions to the total number of mammary glands at risk. Table 2 shows the incidence of mammary lesions in the various groups treated with vitamin D analogues. There was a dose-related decrease in the number of glands exhibiting lesions in the vitamin D₃-treated group. In contrast, in the group treated with 0.01 µM vitamin D₃, only two of 14 glands developed lesions. At higher concentrations of this analogue, no mammary lesions were observed. We calculated the percent inhibition of formation of lesions in each treatment group by comparing the incidences of lesions between the control group and the treatment group. As shown in Fig. 3, both 1α(OH)D₃ and 1α,25(OH)₂D₃ inhibited the formation of mammary lesions by nearly 100%. At a concentration of 0.01 µM, the vitamin D₃ analogue inhibited mammary alveolar lesion formation by 75%; incubation of glands with concentrations of 0.1 µM and higher showed 100% inhibition. In contrast, the vitamin D₃ analogue inhibited the lesion formation in a dose-dependent manner, reaching 100% inhibition at a concentration of 1.0 µM. 1α,25(OH)₂D₃ was toxic to the glands at concentrations of 1.0 µM or higher. Dilution of ducts and disintegration of the morphologic structures were observed. Treatment of mammary glands with 1α(OH)D₃ did not result in any toxicity to the glands.

To determine the effects of vitamin D analogues on the structural differentiation as well as their toxic effects on mammary glands, we incubated mammary glands with growth-promoting hormones for 3 days either alone or in the presence of 0.1 µM or 1.0 µM vitamin D analogues. The histopathologic characteristics of the treated and untreated glands are shown in Fig. 4. The control mammary gland structure was represented by normal alveolar and ductal structures (Fig. 4, A and B, respectively). 1α,25(OH)₂D₃ at a concentration of 0.1 µM did not show toxicity (Fig. 4, C). Mammary glands displayed normal ductal and alveolar structures. At a concentration of 1.0 µM, vitamin D₃ analogue treatment resulted in disintegration of ducts and structural toxicity to the glands (Fig. 4, D). In contrast, treatment with the vitamin D₃ analogue at a concentration of 1.0 µM retained the healthy structural characteristics, as seen in the untreated glands. In fact, some secretion was obvious in the lumen of the ducts (Fig. 4, E).

Since the role of chemopreventive agents (including analogues of vitamin D₃ and vitamin D₄) on the induction of TGF-β in normal mammary epithelial cells has not been studied, the histologic sections of normal mammary glands treated with either only hormones (insulin, progesterone, aldosterone, and hydrocortisone) or hormones plus vitamin D analogues were processed immunohistochemically to investigate the effects of

Table 2. Effects of vitamin D analogues on incidence of 7,12-dimethylbenz[a]anthracene-induced lesions in BALB/c mouse mammary glands in organ culture

<table>
<thead>
<tr>
<th>Concentration, µM</th>
<th>No. of glands with lesions/total</th>
<th>% incidence*</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>9/15</td>
<td>60.0</td>
<td>.21</td>
</tr>
<tr>
<td>0.01</td>
<td>6/16</td>
<td>37.5</td>
<td>.048</td>
</tr>
<tr>
<td>0.1</td>
<td>4/16</td>
<td>25.0</td>
<td>.011</td>
</tr>
<tr>
<td>0</td>
<td>2/14</td>
<td>14.3</td>
<td>.003</td>
</tr>
<tr>
<td>0.5</td>
<td>0/15</td>
<td>0.0</td>
<td>.003</td>
</tr>
</tbody>
</table>

*Number of glands with lesion/total number of glands treated) x 100.
†Two-sided test. Statistical significance when compared with control group.

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vitamin D analogues on the induction and localization of VDRs and TGF-β1. As shown in Fig. 5, VDRs were localized in the nuclei of mammary epithelial cells. There was no selective localization of VDR in ductal or alveolar cells. Treatment with either 1.0 μM 1α(OH)D₃ or 0.1 μM 1α,25(OH)₂D₃ induced expression of VDRs detectable in the nuclei of both ductal and alveolar cells (Fig. 5, B and C, respectively). This induction was dependent on the concentration of the analogue; VDR induction was much less at the lower concentration of the vitamin D₃ analogue (data not shown). For the vitamin D₃ analogue, intense staining was evident at a lower concentration (0.1 μM) (Fig. 5, C). However, at a concentration of 1.0 μM, reduced or absent staining was observed as a result of apparent toxicity (Fig. 5, D). The effects of the vitamin D analogues on the induction of TGF-β1 were also evaluated. We studied tissue sections from the mammary glands treated with the vitamin D analogues or those from untreated control glands for the induction of TGF-β1. We found extensive induction of TGF-β1 in the cytoplasm of mammary epithelial cells (Fig. 6). Again, the pattern of intensity was comparable to that of induction of VDR. The extent of induction of TGF-β1 after treatment with the vitamin D₃ analogue at a concentration of 1.0 μM (Fig. 6, B) was similar to that observed with the vitamin D₃ analogue at a concentration of 0.1 μM (Fig. 6, C). However, at a concentration of 1.0 μM of the vitamin D₃ analogue, TGF-β1 expression was much reduced as a result of toxicity (Fig. 6, D). These results indicate that the vitamin D₃ analogue is considerably less toxic than the vitamin D₃ analogue. Moreover, they indicate that this remarkable induction of TGF-β1 in mammary epithelial cells by the vitamin D₃ analogue may be of importance in cancer chemoprevention.

Discussion

In recent years, considerable attention has been directed to the process of preventing carcinogenesis. The term "chemopreven-
tion" was coined to denote the prevention of cancer by purified and well-characterized chemical agents (37). Numerous chemopreventive agents have been identified that can arrest the process of either initiation or progression of the disease. These agents include retinoids, thiois, inhibitors of polynucleotide biosynthesis and prostaglandin biosynthesis, and antihormones. An active metabolite of vitamin D3, 1α,25(OH)2D3, inhibits the proliferation of many cell types (6-8); moreover, it is a potent inducer of differentiation, as is seen in HL-60 cells (3). However, the use of this analogue to treat cancer patients is precluded because of its calcemic activity. It has been well established that vitamin D treatment results in an increased concentration of calcium in plasma of experimental animals (9). Numerous structural modifications have been reported to generate less calcemic vitamin D analogues with increased antiproliferative or differentiating ability (10).

As this article demonstrates, we synthesized a novel vitamin D3 analogue and determined its calcemic activity. We found that 1α(OH)D2 exhibited lower toxicity than 1α,25(OH)2D3. Nonetheless, 1α(OH)D2 is not completely devoid of calcemic activity. There are at least two specific vitamin D3 analogues, Ro24-5531, a hexafluoro derivative of vitamin D3 (28), and 22-oxa-calcitriol (32), that have been shown to be effective against mammary carcinogenesis. Ro24-5531 is non-calcemic and is capable of inhibiting carcinogen-induced mammary lesions in organ culture; however, it was toxic at a concentration of 0.1 μM in organ culture (data not shown), and the toxicity was observed when the analogue was given in vivo at a dose of 10 μg/kg diet. Since several hundred analogues of vitamin D3 have been evaluated without much success (10), the logical step toward finding an ideal compound was to evaluate the vitamin D analogues found within the D4, D5, and D6 series.

Various reports (33,34) have shown that TGF-β1 is a negative growth regulator for tumor cells. Increased expression of TGF-β1 is directly related to a reduced rate of growth of cancer cells in vitro. Several chemopreventive agents induce TGF-β1 in vitro in transformed cells, including breast cancer cells (25). In a report (28), a non-calcemic analogue of vitamin D3, Ro24-5531, induced expression of TGF-β1 and its type 2 receptors. Similarly, chemopreventive agents have been shown to induce TGF-β1 in breast cancer cells in culture (34). There is no report, however, yet indicating induction of TGF-β1 in normal mammary epithelial cells. Induction of TGF-β1 by chemopreventive agents in normal cells may be more relevant to chemoprevention.
than induction of TGF-β1 in transformed cells. In this study, we have shown that TGF-β1 could be induced by 1α(OH)D₃ in mammary ductal cells and mammary alveolar cells.

Numerous studies have reported that the action of vitamin D is mediated by nuclear VDRs. Our results are consistent with those published (19-21,23). Our study shows that the analogue of the vitamin D₃ series induces nuclear VDRs in the mammary glands in vitro culture. Both 1α(OH)D₃ and 1α(OH)D₃ induced VDRs in mammary epithelial cells. The induction of VDR with 1.0 μM 1α(OH)D₃ was much greater than with the nontoxic concentration of 1α,25(OH)₂D₃. Since the mammary epithelial cells (e.g., HBL-100), which lack VDRs, also fail to respond to 1α(OH)D₃ and do not show induction of TGF-β1 (data not shown), the results presented in this article indicate that the chemopreventive activity of the vitamin D analogue may be mediated by induction of TGF-β1 in mammary tissue and that the vitamin D action may require VDRs. These results also point out that this remarkable induction of TGF-β1 in mammary epithelial cells by vitamin D analogues may be of significant importance in cancer chemoprevention.

Experiments presented here constitute the first step toward our long-term goal of investigating the efficacy of chemoprevention by and the mechanism(s) of action of analogues of the vitamin D₃ series of compounds. Reduced calcemic activity and lack of toxicity make 1α(OH)D₃ an attractive candidate for in vivo chemoprevention studies.

References


Notes

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