Vitamin D and Retinoblastoma

The Presence of Receptors and Inhibition of Growth In Vitro

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The vitamin D receptor has been found in several human organs not involved in calcium metabolism and in several malignant neoplasms found in humans. The role of the receptor in these tissues is unclear. There is, however, a relationship between the presence and quantity of the vitamin D receptor in a malignant cell line and the antineoplastic effect of vitamin D on that cell line. We found that Y-79 retinoblastoma cells have receptors specific for calcitriol (1,25-dihydroxycholecalciferol). Scatchard analysis of the receptor data shows a quantity of 56,000 receptors per retinoblastoma cell. These receptors have a dissociation constant of 1.18 nmol/L. Retinoblastoma cells treated with 10^-8 mol/L of calcitriol for nine days had 15% less cell growth than the control cells. Further studies of the effect of vitamin D on retinoblastoma may warrant its inclusion in chemotherapeutic protocols for the treatment of this childhood affliction.

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Vitamin D plays an important role in the maintenance of calcium homeostasis.1 Abundant vitamin D receptors are present in the intestine, skin, bone, and kidney—all of which play a role in regulating serum calcium levels.1 Interestingly, vitamin D receptors are also present in several organs that are not involved in calcium metabolism, such as the retina, pancreas, pituitary, muscle, female reproductive organs, brain, bone marrow, and thymus.2,3 The role of the vitamin D receptor in normal tissues not involved in calcium metabolism is unclear. Researchers have speculated, however, that vitamin D may act as a general promoter of cell maturation.4 Several malignant neoplasms in humans have 1,25-dihydroxycholecalciferol receptors, such as breast, colon, renal, lung, and cervical cancer; malignant melanoma; histiocytic lymphoma; and myeloid leukemia.5,6 In vitro, nanomolar concentrations of calcitriol (1,25-dihydroxycholecalciferol) induce differentiation and inhibit growth of human leukemia, breast cancer, malignant melanoma, and histiocytic lymphoma cells.7,8,9 Calcitriol also inhibits phorbol ester-dependent chemical carcinogenesis in mouse skin.8

Evidence exists that the antineoplastic effect of vitamin D is receptor mediated.8,10 The vitamin D receptor has a site that binds DNA and affects gene expression.1 Vitamin D, and vitamin D, are the two common forms of vitamin D found in nature. Although both forms are found in our diet, only vitamin D, can be synthesized by a photochemical reaction in our skin.17 Calcitriol has the highest affinity for the vitamin D receptor, and vitamin D, which is not hydroxylated, is virtually inactive.18 In this article, we show that Y-79 retinoblastoma cells have calcitriol receptors and that the cells are inhibited in vitro by calcitriol.

MATERIALS AND METHODS

Cells and Cell Culture

Y-79 retinoblastoma cells10 that had been maintained in nude mice were cultured at 37°C in an atmosphere of 5% carbon dioxide and 95% air using Ham's F-12 media supplemented with 15% fetal bovine serum (volume per volume).

Calcitriol Receptor Assay

Tritiated calcitriol (99,400 cpm = 1 pmol) was dissolved in 95% alcohol at concentrations of 30, 15, 7.5, 3.8, 1.9, 0.5, 0.2, 0.1, and 0.06 nmol/L; 0.2 mL of each tritiated vitamin D solution was pipetted into three test tubes. The alcohol was evaporated from each tube under nitrogen. Then 10^4 Y-79 retinoblastoma cells suspended in 0.2 mL of Hank's balanced salt solution (HBSS, Gibco) were added to each tube; 1.0 ng of cold calcitriol was added to one tube at each concentration. The tube with the cold calcitriol was used to determine nonspecific binding.

The cells were incubated for one hour at 37°C. The tubes were vortexed at the beginning of the incubation and at ten-minute intervals. The cells were diluted with 1.0 mL of HBSS and vortexed and centrifuged at 2000 g for 15 minutes. The supernatant was poured off and counted for radioactivity. One milliliter of HBSS was then added to the remaining pellet, and the cells were resuspended, vortexed, and centrifuged at 2000 g for 15 minutes. The supernatant was again poured off and counted, and the remaining pellets of cells were reacted with 0.1% Triton X-100 (Sigma) in 1.0 mL of HBSS for 30 minutes at 37°C. During this incubation, cells were vortexed at 0, 15, and 30 minutes. Cells were then
centrifuged for 15 minutes at 2000 g, which separated the lysate (cytosol) from the cell nuclear membranes. The lysate and pellet were then counted separately.

The tritiated calcitriol that emitted counts from the lysate and pellets from tubes incubated with cold calcitriol added was considered nonspecifically bound. The tritiated calcitriol that emitted counts from the lysate and pellets from the other tubes was considered both specifically and nonspecifically bound. The tritiated calcitriol that emitted counts from the two supernatant washings was considered unbound or free.

Scatchard analysis of these data was performed to determine the dissociation constant of the calcitriol receptors and the quantity of receptors per cell.

**Growth Assay**

The vitamin D compounds were dissolved in 95% alcohol (VWR, Omni-Solve). Stock solutions of 10⁻⁴, 10⁻³, and 10⁻¹² mol/L of ergocalciferol and calcitriol were prepared by diluting the alcohol solution in media supplemented with 10% dialyzed fetal bovine serum. Stock solutions had a final alcohol content of 0.1% and were stored at −20°C in the dark under argon. All manipulations using the vitamin D compounds were performed in subdued lighting. Since vitamin D in solution has a short shelf life, new stock solutions were prepared every ten days.

Two days prior to treatment, 5 × 10⁴ cells per milliliter were seeded in replica 25-cm² Falcon flasks. On the first day of treatment, three samples of cells from each of ten flasks were counted under a hemocytometer to determine seeding efficiency. Throughout the study, only viable cells, as determined by the trypan blue exclusion test, were counted.

Every three days the old media were discarded and the cells were treated with either 10⁻¹², 10⁻³, or 10⁻¹² mol/L of ergocalciferol or calcitriol dissolved in fresh media supplemented with 10% dialyzed fetal bovine serum. Cell counts were performed at three-day intervals. Three counts were made for each of the three flasks at each dose.

**RESULTS**

**Calcitriol Receptors**

Scatchard analysis of the receptor assay (Fig 1) showed a concentration of 94 fmol of calcitriol receptors per 1 million retinoblastoma cells, or 56,000 receptors per cell. These receptors have a dissociation constant of 1.18 nmol/L.

**Growth Assay**

Concentrations of 10⁻⁴ and 10⁻³ mol/L of calcitriol were effective at inhibiting the growth of the retinoblastoma cells in vitro (Figs 2 and 3). This inhibition was statistically significant at 10⁻¹² mol/L of calcitriol on day 6 and at 10⁻³ mol/L of calcitriol on day 9 (P < .05). The 10⁻¹² mol/L of calcitriol did not statistically significantly inhibit cell growth. By day 9 of the study, cells treated with 10⁻¹² mol/L of calcitriol had proliferated more than the control cells and had a 10% greater cell count than the control cells. The inhibition of cell growth was dose dependent on days 3 and 6. Due to an increased growth rate of the cells treated with high-dose calcitriol compared with control cells between days 6 and 9, the inhibition by calcitriol was not dose dependent on day 9.

Throughout the nine days of the study, growth of the retinoblastoma cells treated with ergocalciferol was similar to or greater than that of the untreated control cells. By day 9 of the study, the cell counts in flasks treated with 10⁻¹², 10⁻³, and 10⁻¹² mol/L of ergocalciferol were 104%, 96%, and 127% of those of the control cells. Ergocalciferol is the inactive, nonhydroxylated precursor of 1,25-dihydroxycholecalciferol, an analogue of calcitriol. Therefore, its inability to inhibit cell proliferation was expected. The promotion of cell growth by high-dose ergocalciferol, however, was unexpected and cannot be explained at this time.

**COMMENT**

These studies show that human Y-79 retinoblastoma cells contain a high-affinity receptor specific for calcitriol. The K_d of this receptor is similar to that described for calcitriol receptors in several other receptor-positive malignant cell lines. Most researchers have found that the growth of cells with receptors is inhibited by vitamin D in proportion to the quantity of receptor present and the affinity of the receptor for the vitamin D compound tested. There are, however, reports of neoplastic cell lines with calcitriol receptors that are not inhibited by calcitriol. The Y-79 retinoblastoma has a higher concentration of receptors per cell than malignant melanoma and colonic, pancreatic, and bladder carcinoma. The vitamin D receptor of the Y-79 retinoblastoma has a lower affinity for calcitriol than the receptors described for the above malignant neoplasms.

Therefore, our receptor studies suggest that the retinoblastoma should be markedly inhibited by calcitriol. Retinoblastoma cells treated for nine
days with $10^{-2}$ and $10^{-9}$ mol/L of calcitriol yielded 15% fewer cells than the control cells (Fig. 3). Other researchers have shown that several receptor-positive human melanoma and breast cancer cell lines treated with $10^{-4}$ mol/L of calcitriol yielded 30% to 50% fewer cells than the control cells.\(^{15}\) The mild inhibition of retinoblastoma cell growth in vitro by calcitriol does not necessarily imply a mild inhibition of the tumor growth in vivo by this agent. The quantity and composition of the nutrient solution used to supplement the tissue culture growth media can affect the actions of calcitriol in vitro.\(^{16}\) In fact, studies in this laboratory have shown that the growth of retinoblastomas in vivo is markedly inhibited by calcitriol.

This article discussed the presence of a receptor specific for calcitriol in retinoblastoma cells and inhibition of growth of these cells in vitro by calcitriol. The human retinoblastoma cell line was not inhibited by ergocalciferol, which is an inactive, nonhydroxylated analogue of calcitriol. We have also shown that calcitriol inhibits the growth of Y-79 retinoblastomas in vivo. Since retinoblastoma cells have receptors for calcitriol, the cells should be sensitive to any antineoplastic drugs that exploit the presence of this receptor. Further studies investigating the effectiveness of calcitriol against retinoblastoma may lead to the use of vitamin D or analogues of vitamin D in treatment protocols for this childhood cancer.

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References