Vol. 179, No. 1, 1991
August 30, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 57-62

ANTIPROLIFERATIVE EFFECT OF 1,25-DIHYDROXYVITAMIN D_3 AND ITS ANALOGS ON HUMAN COLON ADENOCARCINOMA CELLS (CACO-2): INFLUENCE OF EXTRACELLULAR CALCIUM

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Received July 7, 1991

SUMMARY. Depending on culture in either "low Ca^{++}" (0.25 mM) or "normal Ca^{++}" (1.8 mM) medium, human colon adenocarcinoma-derived CaCo-2 cells exhibit differential sensitivity to the antiproliferative action of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and of two side-chain modified analogs, 1,25S,26-trihydroxy-Δ22-vitamin D_3 (Ro 23-4319) and 1,25-dihydroxy-Δ16-23yne-vitamin D_3 (Ro 23-7553). CaCo-2 cells cultured under low Ca^{++} conditions exhibit a high proliferative potential, and in these cells, all vitamin D compounds under investigation significantly inhibit [^{3}H]thymidine incorporation into cellular DNA at $\geq 10^{-10}$ M. The rank order of biopotency is: Ro 23-7553$\geq$Ro 23-4319$>1,25(OH)_2D_3$. At 1.8 mM Ca^{++}, only Ro 23-7553 is able to inhibit proliferation of CaCo-2 cells. Parallel to their antiproliferative action, all three vitamin D compounds stimulate akaline phosphatase activity in CaCo-2 cells, indicating their ability to induce differentiated functions at the same time as they reduce neoplastic cell growth.

1,25-dihydroxyvitamin D_3, the hormonal form of vitamin D_3, plays a pivotal role in calcium and inorganic phosphate (Pi) homeostasis through its well established actions on calcium and Pi movements in intestine, kidney and bone. These biological effects are mediated by binding of the hormone to an intracellular high-affinity receptor in a manner analogous to other steroid hormones (1). However, this specific receptor for 1,25-dihydroxyvitamin D_3 has not only been found in cells of the aforementioned typical target tissues but also in a wide variety of other normal or neoplastic cells (see e.g. 2, 3). Earlier observations that 1,25-dihydroxyvitamin D_3 was able to suppress proliferation and induce differentiation of human (4) and murine (5) leukemia cells had stimulated a number of investigations of this new function of the hormone, namely control of proliferation and differentiation, in several other malignant cell lines (e.g. 6). Although the antiproliferative effect of the vitamin D hormone was thereby well established, the feasibility of its therapeutical application as anti-tumor agent apparently is limited by the severe hypercalcemic effects which are seen at the dose level of 1,25-dihydroxyvitamin D_3 required for an effective antiproliferative action. Thus, efforts were made to synthesize analogs of the hormone with similar differentiation-inducing potency but reduced calcium-mobilizing activity (see e.g. 7).

0006-291X/91 $1.50

The intestine, as a main target organ of 1,25-dihydroxyvitamin D_3, has long been speculated to be also a site of the antiproliferative, differentiating action of the hormone (8). Interestingly, recent epidemiologic studies have implicated mild vitamin D deficiency and/or chronic negative calcium balance in the pathogenesis of colorectal cancer, which is one of the most common malignancies in the Western world (9). However, a distinct role of the steroid hormone in the prevention of neoplastic change of colonic epithelial cells was not yet established.

Since intracellular calcium is believed to play a key role in proliferation control in a wide variety of cells (10), and, in particular, elevated extracellular calcium is known to inhibit proliferation of a number of epithelial cell types (11), we studied the effects of 1,25-dihydroxyvitamin D_3 and of two side-chain modified analogs with low hypercalcemic potential (12), 1,25S,26-trihydroxy-Δ22-vitamin D_3 (Ro 23-4319) and 1,25-dihydroxy-Δ16-23yne-vitamin D_3 (Ro 23-7553), on proliferation and differentiation of human colon adenocarcinoma-derived Caco-2 cells in dependence of extracellular calcium levels. For this type of studies this cell line is an ideal model system since CaCo-2 cells have vitamin D receptors (13) and are able to differentiate in culture under certain conditions (14).

MATERIALS AND METHODS

Culture media. Dulbecco's modified Eagle's medium (DMEM) was supplemented with 4 mM glutamine, 10% fetal calf serum (heat-inactivated at 56° C for 30 min), 20 mM HEPES, 50 U/ml penicillin and 50 µg/ml streptomycin. The final Ca^{++} concentration in this medium, designated "normal Ca^{++} medium", was 1.80 mM (as determined by fluorescence titration with a Nova 9 Calcium Analyzer). When the same additions were made to a specially prepared Ca^{++}-free DMEM (Gibco), the total Ca^{++} concentration in this "low Ca^{++} medium" was 0.25 mM.

Cell Culture. Caco-2 cells were routinely cultured in Costar vented tissue culture flasks at 37° C in a humidified atmosphere of 95% air and 5% CO_2 in normal Ca^{++} DMEM. Cultures were refed every 48 h and subcultured serially when approximately 80% confluent. Cells used in experiments were between passages 10 and 50.

Cell Proliferation Assays. 30.000 cells/ml were routinely seeded in 35 mm Falcon plastic tissue culture dishes (Becton-Dickinson Labware). Vitamin D compounds dissolved in ethanol were added at indicated concentrations on day 1 of culture (day of seeding = day 0). Ethanol concentration in all cultures was 0.1%. Fresh media and hormones were added every 48 h. 1,25-dihydroxyvitamin D_3 was a gift of Hoffmann-La Roche, Basle, Switzerland. The vitamin D analogs, 1,25S,26-trihydroxy-Δ22-vitamin D_3 (Ro 23-4319) and 1,25-dihydroxy-Δ16-23yne-vitamin D_3 (Ro 23-7553) were generously provided by Dr. Milan Uskokovic, Hoffmann-La Roche, Nutley, N. J., USA.

DNA synthesis was assessed by measuring incorporation of [^{3}H]thymidine into cellular DNA. Cells were incubated at 37° C in the presence of hormones for 4 h in the respective normal Ca^{++} or low Ca^{++} DMEM containing 4 µCi/ml of [^{3}H]thymidine. Cells were then washed two times with PBS for 2 min and subsequently fixed and extracted twice (10 min each time) with 5% trichloroacetic acid. After two washes with distilled water (5 min each), cells were solubilized in 1 ml of 1 N NaOH. After neutralization with 1 M acetic acid, the extracts were counted for radioactivity. Results are expressed as "percent of hormone-free control", which was set to 100%. Data are shown as means ± SEM from at least three experiments, with each assay done in triplicates.

Cell counting. Cells were trypsinized, diluted and counted in triplicate in a Bürker-Türk haemocytometer on indicated culture days. Viability of cells was ≥90%, as determined by trypan blue exclusion.

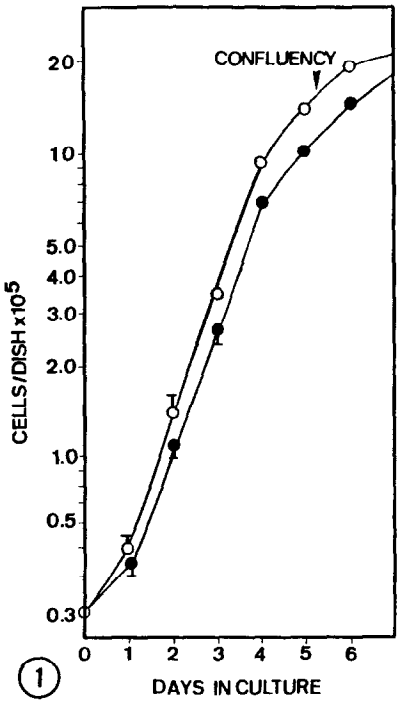
Alkaline phosphatase (E.C. 3.1.3.1) assay. Cells were harvested at day 4. Culture dishes were rinsed twice with 5 ml of PBS and scraped with a "rubber policeman" into 2.0 ml of icecold PBS. Cells were homogenized in an Ultra-Turrax T25 (IKA Labortechnik, Staufen,

FRG) (3x5 s at 8000 rpm and 3x10 s at 21000 rpm). Triton X-100 was added to the homogenate (final concentration 0.1%). Alkaline phosphatase activity was determined by the method of Forstner et al. (15).

RESULTS

Comparison of cell growth in 0.25 mM Ca^{++} to 1.8 mM Ca^{++} DMEM indicated already on day 2 of culture, that Caco-2 cells proliferate more readily in the low Ca^{++} medium. After 5 days, a 30% increment in cell numbers due to growth under low Ca^{++} conditions was observed (Fig. 1). Increased proliferative capacity of CaCo-2 cells in low Ca^{++} as compared to normal Ca^{++} medium was also indicated when [^{3}H]thymidine incorporation into DNA was assayed in parallel cultures (data not shown).

The antiproliferative potency of vitamin D compounds was evaluated from the extent of [^{3}H]thymidine incorporation into cellular DNA after 4 days exposure to the vitamin D sterols, when cells were approaching confluency, but were still in the logarithmic growth phase. During culture with 0.25 mM Ca^{++}, 1,25$(OH)_2D_3$ as well as both analogs significantly inhibited DNA synthesis in Caco-2 cells (Figs. 2-4). Notably, maximum inhibition achieved by 10^{-7} M Ro 23-4319 or 10^{-8} M Ro 23-7553, respectively, was approximately 40%, and was thus much greater compared to the active vitamin D metabolite which at its saturating concentration of 10^{-8} M suppressed [^{3}H]thymidine incorporation only by 20%. In addition, it is obvious from the



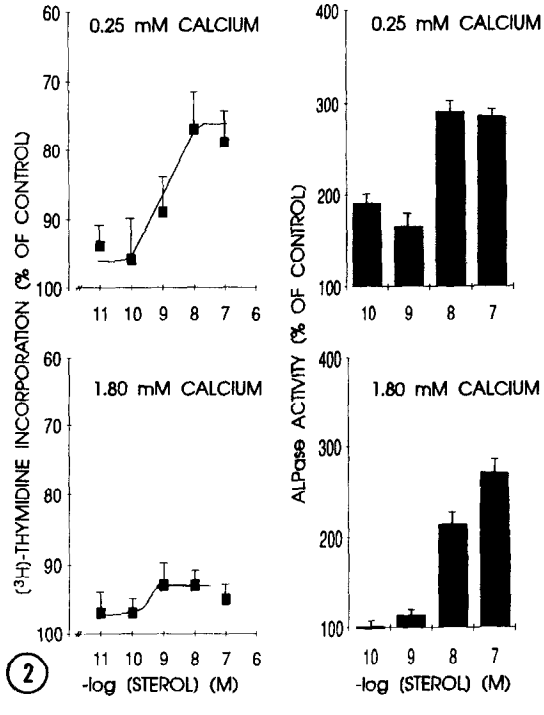


Figure 1. Effect of extracellular Ca^{++} concentrations on growth rate of CaCo-2 cells. ○, 0.25 mM Ca^{++}; ●, 1.8 mM Ca^{++}. Data are means ± S.E.M. (n=4). Error bars are shown where larger than symbols.

Figure 2. Effect of 1,25$(OH)_2D_3$ on proliferation and alkaline phosphatase activity of CaCo-2 cells.

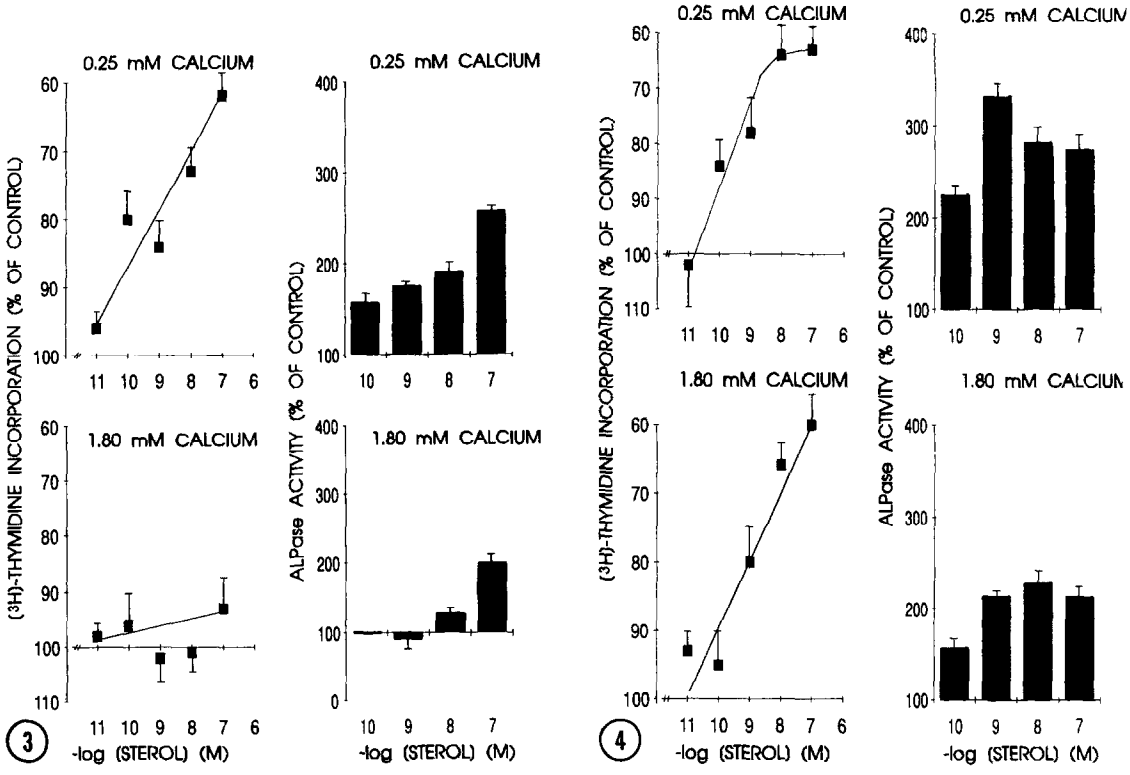


Figure 3. Effect of Ro 23-4319 on proliferation and alkaline phosphatase activity of CaCo-2 cells.
Figure 4. Effect of Ro 23-7553 on proliferation and alkaline phosphatase activity of CaCo-2 cells.

dose-response curves shown in Figs. 2-4, that both analogs were at least 10 times more efficient than $1,25(OH)_2D_3$ when CaCo-2 cells were grown in low Ca^{++} DMEM: The same response as to 10^{-8} M $1,25(OH)_2D_3$ could be elicited by Ro 23-4319 or Ro 23-7553, respectively, already between 10^{-10} - 10^{-9} M.

A markedly different pattern of antiproliferative responses to $1,25(OH)_2D_3$ and the two analogs was observed, when [^{3}H]thymidine incorporation into DNA was evaluated in CaCo-2 cells cultured in 1.8 mM DMEM. Exposure to normal Ca^{++} levels completely abolished the ability of $1,25(OH)_2D_3$ as well as of Ro 23-4319 to inhibit cell growth (Figs. 2 and 3), while Ro 23-7553 still exhibited an inhibitory effect, which was not changed in its magnitude by raising the medium calcium concentration from 0.25 mM to 1.8 mM. However, it seems that thereby the threshold concentration for the antiproliferative action of this analog increased from 10^{-10} to 10^{-9} M (Fig. 4).

Although alkaline phosphatase activity was found to be increased by all three vitamin D compounds, regardless whether Caco-2 cells were grown in low Ca^{++} or normal Ca^{++} DMEM (Figs. 2-4), it is obvious that the reponse to the sterols is attenuated by 1.8 mM Ca^{++}. In general, the magnitude of the effect of saturating sterol concentrations is distinctly lower at the normal Ca^{++} as compared to the low Ca^{++} concentration. In addition, compared to 0.25 mM Ca^{++}, at 1.8 mM a 100fold higher concentration of 1,25(OH)2D3 or of Ro 23-4319, respectively, is required for a significant effect on alkaline phosphatase activity. In case of Ro

23-7553, this shift of threshold concentrations is not observed to the same extent. Therefore, Ro 23-7553, at normal as well as low calcium concentrations in the culture medium, exceeds the other vitamin D compounds under investigation in their potency to induce alkaline phosphatase activity in CaCo-2 cells.

DISCUSSION

In normal epithelial cells low extracellular Ca^{++} typically promotes cell proliferation whereas higher Ca^{++} concentrations elicit terminal differentiation (cf. 10). However, it was suggested that preneoplastic intestinal cells loose the ability to respond to the differentiation signal of high extracellular Ca^{++} by the time they evolve to an advanced benign tumor state as seen, e.g., in villous adenomas (16). Our study demonstrates, however, that the proliferation rate of CaCo-2 cells can be promoted by using 0.25 mM instead of 1.8 mM Ca^{++} DMEM. This strongly suggests that the normal growth inhibitory response to the Ca^{++} signal is not lost in Caco-2 cells, which were originally derived from a human colon adenocarcinoma.

A number of *in vivo* studies (cf. among others, 9) tried to link deficient endogenous vitamin D production and Ca^{++} absorption to the occurrence of colon cancer. However, evidence that the active vitamin D metabolite in a physiological concentration range inhibits growth of colon cancer cells was lacking up to now. In the present study we were able to demonstrate an inhibitory effect of $1,25(OH)_2D_3$ on growth of CaCo-2 cells in 0.25 mM Ca^{++} DMEM at a concentration as low as 10^{-9} M. Thus, $1,25(OH)_2D_3$ can inhibit growth of colon cancer cells at concentrations lower than those used in a leukemia cells assay system (12). The inhibitory capacity of $1,25(OH)_2D_3$ is surpassed by far by the analogs Ro 23-7553 and Ro 23-4319: they are effective in a concentration range (10^{-11} - 10^{-10} M), which is not too far above physiological serum levels of $1,25(OH)_2D_3$ (approximately $5x10^{-12}$ M).

It is highly interesting that particularly CaCo-2 cells with a high proliferative potential, i.e., when their growth rate is stimulated by low extracellular Ca^{++}, exhibit a general sensitivity to the antiproliferative action of vitamin D sterols. However, with respect to a possible use of vitamin D sterols for treatment of colorectal cancers one should keep in mind that at a normal extracellular Ca^{++} concentration, only Ro 23-7553 significantly inhibited proliferation at concentrations lower than 10^{-9} M.

A similar pattern of effectiveness of the vitamin D sterols under investigation was observed when their potency to induce alkaline phosphatase activity in CaCo-2 cells grown at two different medium Ca^{++} concentration was assessed. Since expression of enzyme activity increases during terminal differentiation of colonic epithelial cells (17), we conclude from our results that in CaCo-2 cells growth inhibition induced by $1,25(OH)_2D_3$ as well as by its analogs, Ro 23-4319 and Ro 23-7553, is associated with simultaneous appearence of a differentiated cell function.

ACKNOWLEDGMENT

These investigations were supported by Grant No. 159/90 of the Anton Dreher Memorial Fund of the University of Vienna Medical School.

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