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1,25-DIHYDROXYVITAMIN D_3 INHIBITS THE CLONOGENIC GROWTH OF TRANSFORMED CELLS VIA ITS RECEPTOR

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Anchorage-independent growth in soft agar is a unique property of transformed cells which is known to be correlated with tumorigenicity. We report here that 1,25-dihydroxyvitamin D_3 suppresses colony formation by a number of cultured cancer cell lines in soft agar in a dose dependent manner with an ID_{50} of 5-7 x 10^{-10}M. This effect is also achieved with analogues of 1,25-dihydroxyvitamin D_3 in accordance with their binding affinity for the hormone's receptor. Only cells with 1,25-dihydroxyvitamin D_3 receptor protein are inhibited in their colony formation by vitamin D analogs indicating that the hormone receptor complex may be integrally involved in the in vitro suppression of the anchorage-independent phenotype.

The biologic effects of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) include both traditional target organ responses such as intestinal calcium transport and bone mineral resorption, as well as newly recognized actions in cultured cells. The latter include such phenomena as inhibition of monolayer growth (1-3), morphological alterations (1,3), and induction of the 25-hydroxyvitamin D_3-24 hydroxylase catabolizing enzyme (4). Studies have also revealed that $1,25(OH)_2D_3$ can function to induce differentiation of leukemia cells to macrophages (5,6) and prolongs the survival time of nude mice inoculated with M1 leukemia cells (7). The mediators of these actions are thought to be specific intracellular receptor proteins (8,9). These proteins are macromolecules which display molecular weights of approximately 52,000-60,000 daltons and bind the hormone with high affinity (K_d = 10^{-10} M) and selectivity over other vitamin D metabolites. These proteins are DNA binding species (10) and are considered to function analogously

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to other steroid hormone receptors in the nucleus to stimulate the expression of genes coding for steroid induced proteins (11).

Anchorage-independent growth in soft agar is a recognized assay for malignant transformation, since growth of cells in agar has been significantly correlated with tumorigenicity in immunologically suppressed host animals (12). Hamburger and Salmon have postulated that inhibition of colony formation in soft agar is an applicable means of determining the efficacy of potential anticancer drugs (13). We have studied the effects of $1,25(OH)_2D_3$ on the ability of a spectrum of malignant cell lines, including melanoma, breast and bladder carcinoma and osteosarcoma, to form colonies in soft agar. Furthermore, we tested the hypothesis that the $1,25(OH)_2D_3$ receptor mediates putative actions of the hormone on anchorage-independent growth of transformed cells.

MATERIALS AND METHODS

Assessment of Anchorage Independent Growth: Trypsinized single cells were suspended in 0.3% agar (Difco Laboratories, Detroit, MI) in the appropriate cell culture medium containing 20% fetal bovine serum. In 20% fetal bovine serum the endogenous level of $1,25(OH)_2D_3$ is 3.2×10^{-11} M (2); in all experiments the level of $1,25(OH)_2D_3$ or other vitamin D metabolites represents that introduced in addition to this basal level. One-ml aliquots containing 10^4-10^5 cells were pipetted into each 35 mm Petri dish onto a base layer of 1 ml complete medium containing 0.5% agar. Both lower and upper layers contained either the ethanol vehicle or $1,25(OH)_2D_3$ (or analogues) at the appropriate concentration. Plates were incubated for approximately 2 weeks at 37°C, at which time the colonies were counted, utilizing an optical image analyzer (Omicon Fas II; Bausch & Lomb, Rochester, NY) (14). Colonies 50 μm or more in diameter (approximately 30 cells) were scored and 3 to 5 plates of each cell line were assayed for colony formation in each group. Statistical significance was assessed by the Student's *t* test and the data are depicted as the mean ± SEM.

Measurement of the $1,25(OH)_2D_3$ Receptor: To ensure conservation of receptors, whole cell labeling was employed (6). 100 to 150 x 10^6 intact cells were labeled with 2nM $1,25(OH)_2[^3H]D_3$ (158 Ci/mmol) in the presence of 1% serum at 37°C for ninety minutes. Cells were then pelleted and rinsed three times in ice-cold phosphate buffer (0.14 M NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.5) with 0.1% BSA followed by centrifugation. The resulting pellet was resuspended in 5 ml of HPB buffer (0.1 M Tris HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 5 mM dithiothreitol) and after a 20-min incubation, the cells were lysed using Dounce homogenization. The lysate was centrifuged again for 15 min at 1100 x *g* and the pellet was resuspended in 3 ml of KETD-0.3 hypertonic buffer containing 0.3 M KCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4 and 5 mM dithiothreitol, extracted for 15 min and centrifuged again for 5 min at 12,000 x *g*. This high salt extraction (0.3 M KCl) was used to recover the hormone-receptor complex from the nucleus. The supernatant from this extract was then subjected to DNA-cellulose chromatography. Extracts were diluted with KETD-0 to achieve a final concentration of 0.05 M KCl and applied to DNA-cellulose columns under gravity flow. After

extensive washing with KETD-0.1 (~45 ml), a linear gradient of KCl (0.1 to 0.5 M) in KETD was used. Fractions (3 ml) were collected at a flow rate of 1 ml/min and quantitated for radioactivity; counting efficiency was 29% in these experiments. The peak of receptor binding emerges at approximately 0.25 M KCl and the radioactivity integrated to obtain the number of receptor molecules/cell.

RESULTS AND DISCUSSION

To examine the influence of $1,25(OH)_2D_3$ on anchorage-independent growth we initially selected two cell lines reported to be tumorigenic in nude mice, ROS 17/2.8 rat osteosarcoma (15), and 3T6 mouse fibroblast (16). These cell lines were cultured in soft agar containing 20% serum with the addition of $1,25(OH)_2D_3$ at doses from less than physiologic to the pharmacologic level of 10^{-7}M. As illustrated in Fig. 1, a marked, dose dependent inhibition of colony formation was observed in the presence of $1,25(OH)_2D_3$ which displayed an ID_{50} (that dose which inhibits colony formation by 50% of control) of 5 x 10^{-10}M in the case of ROS 17/2.8 and 7 x 10^{-10}M in 3T6. These ID_{50}'s are equivalent to the normal circulating level of $1,25(OH)_2D_3$ in rodents (17) and suggest an involvement of the $1,25(OH)_2D_3$ receptor in this effect. The concentration of $1,25(OH)_2D_3$ required to suppress anchorage independent growth is 10-100 times lower than that which inhibits growth of attached cells in mono-

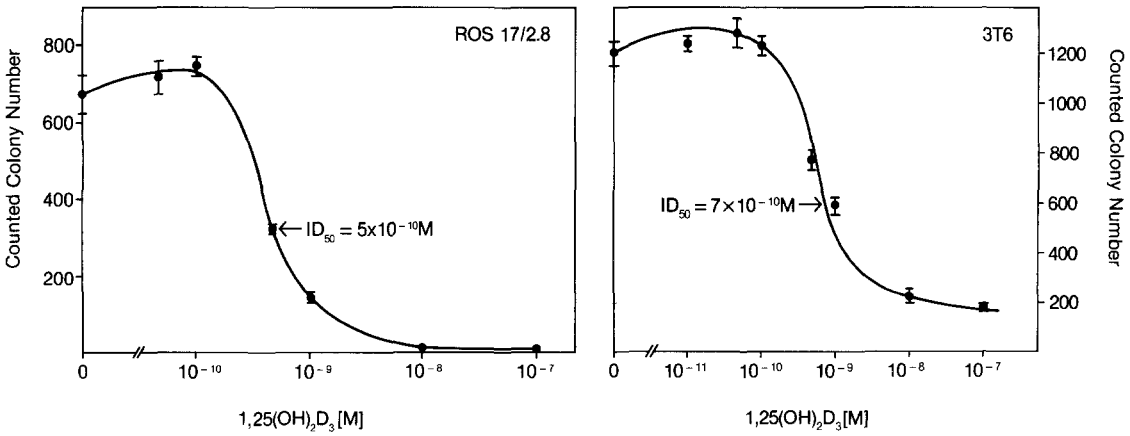

Fig. 1 - Inhibition of cell colony formation in soft agar in the presence of $1,25(OH)_2D_3$. The left panel illustrates the effect of $1,25(OH)_2D_3$ on the colony formation of ROS 17/2.8 rat osteosarcoma cells, while the right panel depicts the results seen using 3T6 mouse fibroblast cells. Note that doses of $\leq 10^{-10}$M $1,25(OH)_2D_3$ cause a slight but insignificant enhancement of colony formation; we have previously reported very small increases in the growth of ROS 17/2.8 and HL-60 cells in monolayer (3) and suspension (6) cultures, respectively, in the presence of such low doses of the hormone.

layer (3). This exquisite sensitivity to the hormone in soft agar could be the result of enhanced responsiveness of colony formation as an unique expression of the malignant phenotype. More likely, the sensitivity is a reflection of this system in which few cells are present to catabolize $1,25(OH)_2D_3$ over an extended period of time.

To ascertain the specifity of the response to $1,25(OH)_2D_3$ we next evaluated the potency of $1,25(OH)_2D_3$ as compared to six other vitamin D analogues utilizing 3T6 cells. A longer acting fluorinated analogue, $24,24F_2$-$1,25(OH)_2D_3$, which binds equally well to the receptor (18) exhibited an inhibition four times more sensitive than $1,25(OH)_2D_3$. Trihydroxy-vitamin D metabolites displayed intermediate potency in inhibiting 3T6 colony formation, with $1,24,25(OH)_3D_3$ being slightly less effective (75%) than $1,25(OH)_2D_3$ and $1,25,26(OH)_3D_3$ having one tenth the activity of the hormone. Of the monohydroxylated D vitamins, $1\alpha(OH)D_3$ showed the highest potency (1/250 that of $1,25(OH)_2D_3$). $24,25(OH)_2D_3$ exerted virtually no activity in this system, probably because of its high binding affinity for serum vitamin D binding protein combined with its low affinity for the intracellular $1,25(OH)_2D_3$ receptor (19,20). The pharmacologic profiles of the various vitamin D metabolites in these experiments corresponded well with the rank order of these metabolites in binding to the receptor (Fig. 2)

Since the data accumulated in both the dose response studies and the analogue investigation suggest that the receptor is an integral part of the action of the $1,25(OH)_2D_3$ hormone in tumorigenic cells, we sought to analyze directly these cells for the existence of the $1,25(OH)_2D_3$ receptor. We utilized the receptor's DNA binding property (10) to assess its presence in a number of lines. Fig. 3A portrays a typical experiment in a tumor cell line, human melanoma G-361. A peak of apparent receptor binding eluted at 0.25 M KCl, the characteristic salt concentration at which the $1,25(OH)_2D_3$ receptor elutes from DNA cellulose (10). Integration of the radioactivity in the peak corresponds to approximately 1900 receptor molecules/cell. In contrast, in Fig. 3B, the IMR-32 human neuroblastoma line revealed an absence of a binding

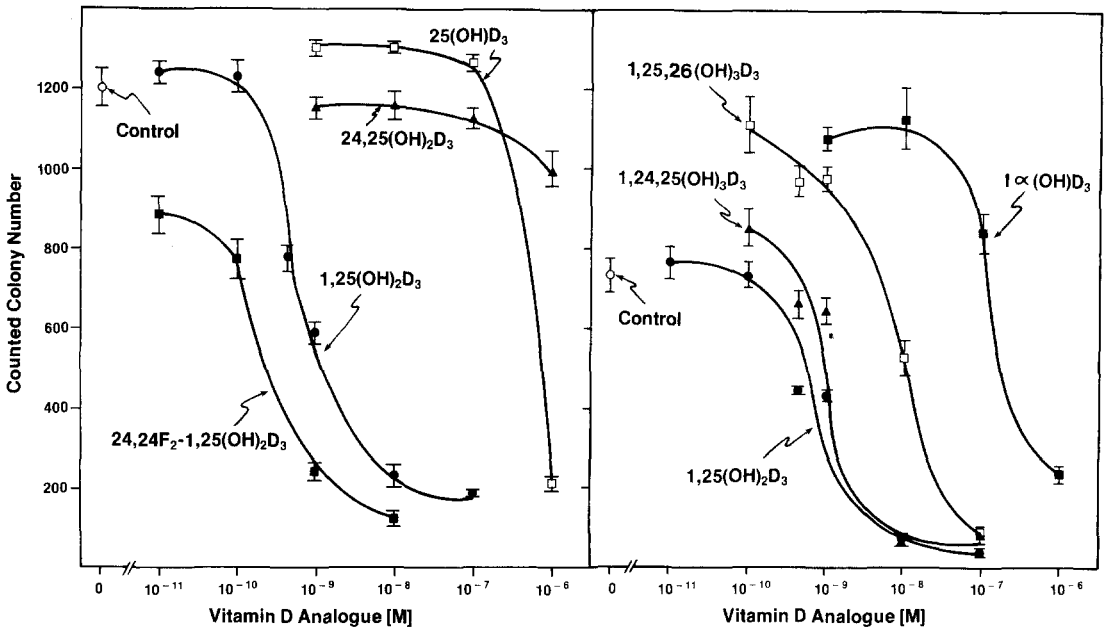

Fig. 2 - Influence of $1,25(OH)_2D_3$ and its analogues on anchorage-independent growth of 3T6 cells. The left panel illustrates the results of $1,25(OH)_2D_3$, a longer acting fluorinated analogue, $24,24F_2$-$1,25(OH)_2D_3$, and other vitamin D metabolites $24,25(OH)_2D_3$ and $25(OH)D_3$; the right panel shows the effects of $1,24,25(OH)_3D_3$, $1,25,26(OH)_3D_3$ and $1\alpha(OH)D_3$.

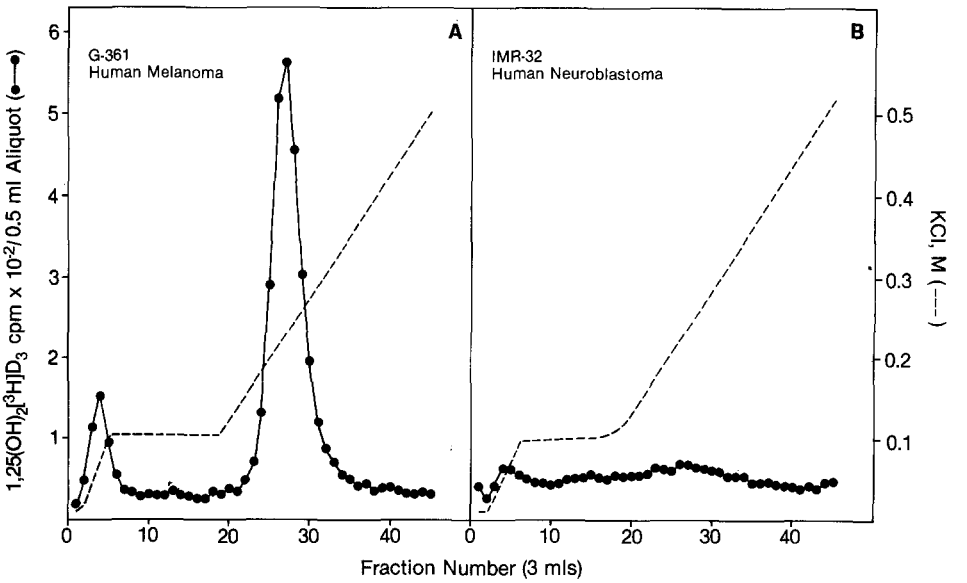

Fig. 3 - DNA cellulose chromatography of $1,25(OH)_2D_3$ receptor in cancer cell lines. The left panel depicts the results of DNA chromatographic analysis of G-361 human melanoma cells while the right panel shows the results with IMR-32 human neuroblastoma.

Table 1. $1,25(OH)_2D_3$ Receptor Presence Correlates with Anchorage-Independent Growth Inhibition

Cell line	$1,25(OH)_2D_3$ Receptor Number/Cell	% Inhibition of Colony Formation
3T6 mouse fibroblast	13,000	95
ROS 17/2.8 rat osteosarcoma	10,000	92
G-361 human melanoma	1,900	52
MCF-7 human breast carcinoma	1,500	41
EJ human bladder carcinoma	1,400	21
ROS 2/3 rat osteosarcoma	100	NS*
ROS 24/1 rat osteosarcoma	<100	NS*
IMR-32 human neuroblastoma	<100	NS*

*NS=Not significant

Receptor number/cell was quantitated by DNA cellulose chromatography as described in Methods. Percent inhibition of colony formation determined in the presence of 10 nM $1,25(OH)_2D_3$ was obtained by comparing treated cells to control cells grown in the presence of 0.1% ethanol vehicle only.

peak on DNA cellulose. These two examples disclose a distinct disparity in the occurrence of the receptor in human tumor cell lines. We examined these and several other cancer cell types for their potential to form colonies in soft agar and have measured their receptor content via DNA-cellulose chromatography. A summary of these data is listed in Table 1 which revealed that $1,25(OH)_2D_3$ was active in suppressing anchorage-independent growth in a spectrum of cancer cells, including melanoma, breast and bladder carcinoma, and osteosarcoma. More importantly, these data delineate an apparent correlation between receptor number and inhibition of colony formation by 10nM $1,25(OH)_2D_3$.

The mechanism by which $1,25(OH)_2D_3$ functions in this system probably involves receptor binding to DNA, an event which apparently alters transcription (21) and may initiate the program for differentiation of transformed cells. This phenomenon could involve the suppression of oncogene expression by the $1,25(OH)_2D_3$-receptor complex as has recently been reported for c-*myc* in

the HL-60 human leukemia line (22). Alternatively, since $1,25(OH)_2D_3$ is a known calcium transport hormone (8), it may be the hormone mediated change in intracellular calcium which triggers cell differentiation (23) and causes the apparent reversion of malignancy.

Suppression of anchorage-independent growth of tumor cells in soft agar has also been demonstrated in several other hormone and vitamin systems, such as estradiol and its antihormone tamoxifen (24), glucocorticoids (25) and the retinoids (26). Although such compounds as these have documented anticancer effects, in vivo, it is premature to propose on the basis of inhibition of colony formation that $1,25(OH)_2D_3$ is a natural anticancer agent. However, preliminary evidence indicates that $1,25(OH)_2D_3$ may indeed be effective, in vivo, by retarding phorbol diester promotion of skin tumors in mice (27). This concept requires extensive verification employing other animal models of neoplasia. In conclusion, the data in this study demonstrate that $1,25(OH)_2D_3$ suppresses anchorage-independent growth of a number of tumorigenic cell lines in soft agar and complement previous findings (7,27) which suggested that $1,25(OH)_2D_3$ has potential antitumor effects. We also show that this action of $1,25(OH)_2D_3$, like the more traditional actions of this hormone, is mediated by its DNA-binding receptor protein.

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