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1,25-DIHYDROXYVITAMIN D_3-INDUCED GROWTH RESTRICTION OF CULTURED EPITHELIAL CELLS DERIVED FROM A MURINE HEPATIC TUMOR*

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Abstract—Recent evidence suggests that 1,25-dihydroxyvitamin D_3 can accumulate in certain presumed non-target tissues, although the mechanism of action of the vitamin in such cells is not understood. Exposure of 77-1/3a mouse hepatic tumor cells, which derived from a non-target tissue of vitamin D action, to 1,25-dihydroxyvitamin D_3 in chemically-defined serum-free medium resulted in a dose-dependent decline in cellular growth rate and maximal culture population density but did not adversely affect cell viability. Culture of 77-1/3a cells in defined medium containing 10^{-7} or 10^{-6} M 1,25-dihydroxyvitamin D_3 for 150 hr reduced the growth rate to 64 and 50% of control values respectively. Albumin secretion was unaffected by 1,25-dihydroxyvitamin D_3 exposure; in contrast, the cellular content of the proliferation-associated protein p35 was reduced by 39%, a decline similar in trend and degree to that observed in other tumor cells exposed to differentiation-inducing agents. It appears that 1,25-dihydroxyvitamin D_3 regulates cellular p35 content (within a specific restricted range) as a consequence of proliferative perturbation, rather than differentiated status, of cultured hepatic tumor cells.

The active metabolite of vitamin D_3, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$§), exerts its pharmacological effect by interacting with a specific cytosolic receptor present in certain target tissues such as intestine, bone and kidney [1, 2]. Many tissues not previously known to be targets for vitamin D action, however, also possess the 1,25-$(OH)_2D_3$ receptor [2]; [^{3}H]-1,25-$(OH)_2D_3$ accumulates in the epithelial cells of both target and presumed non-target tissues as well [3], suggesting that 1,25-$(OH)_2D_3$ may have more widespread pharmacological actions than previously thought. The presence of the 1,25-$(OH)_2D_3$ receptor is a common occurrence in various cancer cells [4, 5] including those of epithelial origin [6]. It has been shown, for example, that 1,25-$(OH)_2D_3$ inhibits proliferation of tumor cell growth [7, 8], induces differentiation of myeloid leukemia cells into monocytes/macrophages [8], and prolongs the survival time of mice inoculated with myeloid leukemia cells [9].

We, therefore, investigated the effect of 1,25-$(OH)_2D_3$ on the growth and expression of differentiated traits of malignant epithelial cells derived from a well-characterized tumor of a presumed non-target organ (liver). The specific established tumor line (murine hepatic carcinoma, BW77-1 clone 3a) was selected for evaluation since it has been shown previously to be responsive to at least one differentiation-inducing chemical agent (e.g. dimethyl sulfoxide, DMSO), resulting in the expression of more highly differentiated cell type-specific traits [10]. 77-1/3a cells were found to exhibit growth restriction in response to pharmacological levels of 1,25-$(OH)_2D_3$ exposure with an associated reduction in the cellular content of p35, a protein which, in murine erythroleukemia cells, is regulated in a proliferation-dependent manner [11].

MATERIALS AND METHODS

77-1/3a cells, a clonal derivative of the BW77-1 hepatic tumor cell line [10, 12], were cultured in serum-supplemented growth medium (SSM) consisting of RPMI 1640 medium containing 10% (v/v) fetal bovine serum and standard concentrations of antibiotics (GIBCO, Grand Island, NY). Cells were either propagated directly in this medium or changed-over to serum-free chemically-defined growth medium (DM) which contained RPMI 1640 base medium and supplemented with (per ml): insulin, 10 μg; glucagon, 10 μg; epidermal growth factor, 50 ng; linoleic acid-albumin complexes, 1 mg (formulation of Enat *et al.* [13]). Authentic 1,25-$(OH)_2D_3$ was the gift of Dr. Uskkovic (Hoffmann-LaRoche, Nutley, NJ). Media were removed from control and 1,25-$(OH)_2D_3$-treated cells, clarified at 13,000 *g*, and stored at −20°. The cells were extracted by sonication in 0.1% Triton X-100 in phosphate-buffered saline, pH 7.2, and insolubles were removed by centrifugation at 13,000 *g*. Equal amounts of cellular extract protein [14] or aliquots of culture media were applied to nitrocellulose mem-

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§ Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; DMSO, dimethyl sulfoxide; SSM, serum-supplemented growth medium; and DM, serum-free chemically-defined growth medium.

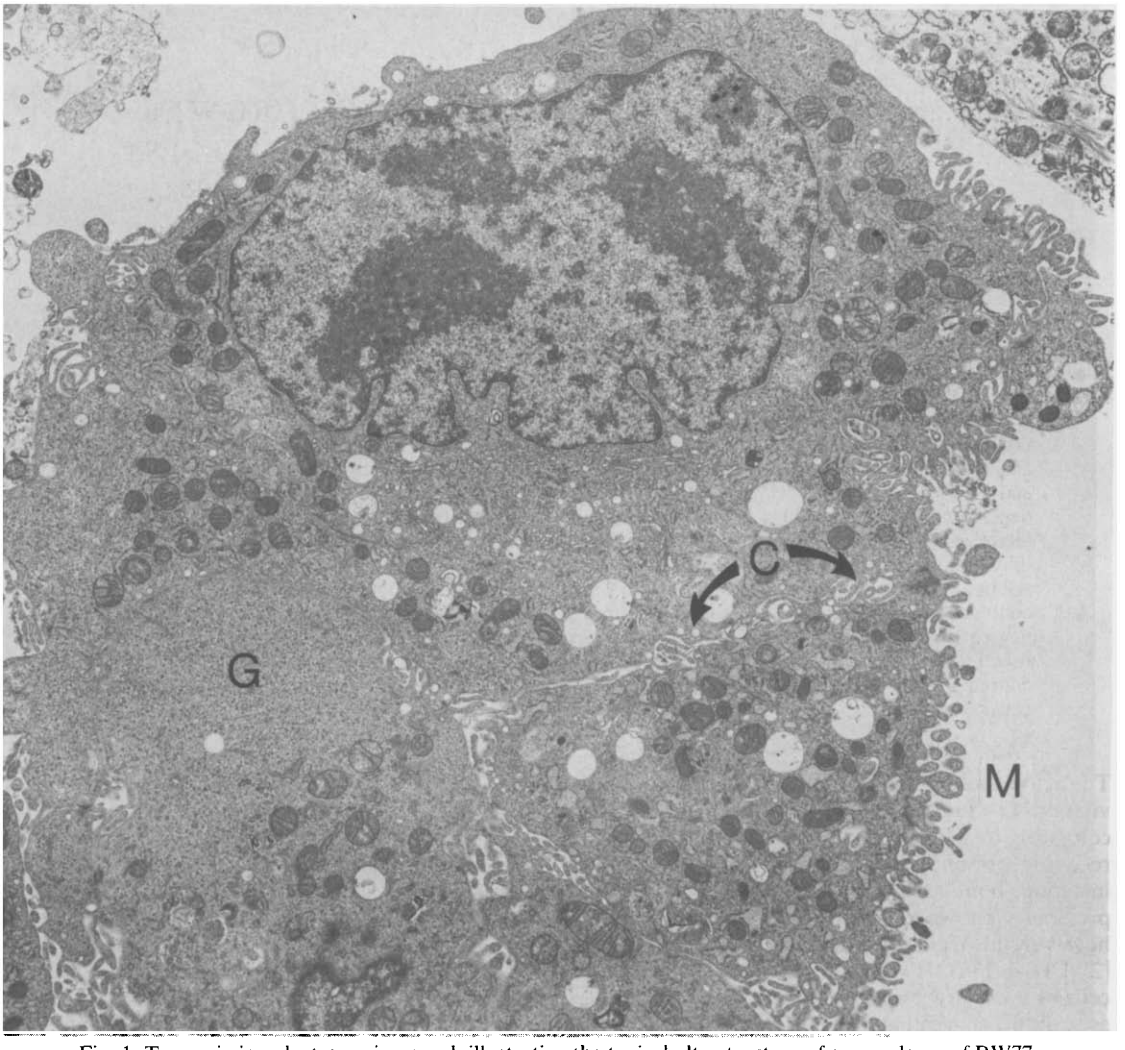

Fig. 1. Transmission electron micrograph illustrating the typical ultrastructure of a monolayer of BW77-1/3a hepatic tumor cells. The available morphologic and functional criteria of BW77-1/3a and related cell lines [19] suggest classification as a primitive hepatocytic cell type. Glutaraldehyde-fixed cell monolayers were rinsed with water and then post-fixed with 2% OsO_4 for 1 hr. Following dehydration, first with 70 and 97% ethanol (each containing 1% uranyl acetate) and then with absolute ethanol, the cells were released as a monolayer by treatment with propylene oxide, centrifuged into the tips of Beem capsules, and embedded in Epon. Thin sections, stained with 5% uranyl acetate in absolute methanol and with lead citrate, were examined with a JEOL/100B electron microscope; ×3000. Key: (M) microvillar border; (C) canaliculus-like structures; and (G) glycogen deposits.

branes using a Bio-Dot apparatus (Bio-RAD, Richmond, CA). Blocking of the free binding sites on the nitrocellulose membrane and probing of the cellular protein or media blots with rabbit antisera to murine p35 and albumin [11, 15, 16], respectively, followed by detection of primary immunoglobulins with peroxidase-conjugated goat-rabbit IgG (Bio-Rad) was done essentially as described previously [17]. The specificity of antisera to p35 and mouse albumin was confirmed by immunoprecipitation analysis [11, 18]. After development of the peroxidase color reaction with diaminobenzidine-tetrahydrochlorine [17], the nitrocellulose blots were scanned with a densitometer. Serial dilutions of electrophoretically-purified mouse albumin [16] were used for standard regression analysis of the albumin content of the cell culture media; p35 content was expressed relative to its level in control cells (100%). Cells in a separate group of treated or untreated cultures were trypsinized into suspension and counted with a hemocytometer in order to determine the cell density in cultures used for albumin quantitation.

RESULTS AND DISCUSSION

The morphology and ultrastructure of the 77-1/3a cell line are generally similar to other established cell lines derived from the transplantable BW7756 murine hepatic tumor [19]. Cultures comprised elongated, triangular-to-goblet shaped, predominantly mononuclear cells of uniform size, each cell possessing 2–3 prominent nucleoli with a

Table 1. Effects of various growth media on population kinetics of 77-1/3a hepatic tumor cells

Medium	Saturation density (cells/culture) ($\times 10^{-6}$)	Population doubling time (hr)	Percent viability
SSM	3.5 ± 0.7	23.1 ± 2.3	93.7 ± 2.9
DM	2.6 ± 0.5	31.3 ± 3.4	96.6 ± 4.9
DM + 50 mM EtOH	2.4 ± 0.4	33.3 ± 3.7	95.7 ± 3.3

Equivalent numbers of 77-1/3a cells were seeded to tissue culture in SSM for 24 hr and then propagated in SSM, DM or DM + 50 mM ethanol (EtOH; the solvent for $1,25\text{-}(OH)_2D_3$) for an additional 7 days; media were changed every second day. At days 2, 3, 4, 5, 6, and 7, cells in triplicate plates from each group were trypsinized into suspension and counted by a hemocytometer; viability was determined by a Trypan Blue dye exclusion assay. Population doubling time was calculated by regression analysis of culture density (cells per culture) during the period of exponential phase growth (days 2–5). Values are means ± SD of triplicate determinations.

nuclear:cytoplasmic ratio of approximately 1. The 77-1/3a cells possessed characteristic surface microvilli, and particular expansions of the intracellular space appeared to have the general morphology of canaliculus-like structures but lacked the rigorous orientation of junctional complexes usually associated with bile canaliculi *in vivo* (Fig. 1). It is concluded that these cells represent a primitive hepatocytic phenotype.

Culture of 77-1/3a cells in SSM for 24 hr followed by change-over to, and subsequent growth in, DM resulted in a decline in maximum population density (to a level approximating 70–75% of that achieved in SSM); this was a typical response of the 77-1/3a line to DM and, in all probability, reflected a reduced proliferation due to the absence (in DM) of certain growth factor(s) usually provided by serum (Table 1). The lowered final population density seen in DM, relative to SSM-propagated cells, was not due simply to loss of cells because of adherence limitations. Cells cultured in DM were fully adherent with no significant increase in "floating" or detached cells relative to SSM controls. Similarly, EtOH (when added to DM at a final concentration of 50 mM) did not alter growth characteristics (i.e. saturation density, doubling time, or viability) of 77-1/3a cells in a manner different from that seen in DM alone. Although 77-1/3a cells grew well in DM, and did so apparently in response (in large measure) to epidermal growth factor (Table 2), they were not able to attach to the culture vessel when seeded directly into DM, requiring 24 hr of exposure to SSM to allow for attachment to occur prior to the change-over to DM. It appears, therefore, that (unlike DM) initial exposure to SSM provides (or allows for the synthesis of) essential matrix factors required by 77-1/3a epithelial hepatic tumor cells (addition of fibronectin at 10 μg/ml to DM reduced the serum requirement for attachment without significantly altering population doubling times).

Culture of 77-1/3a cells in DM containing $1,25\text{-}(OH)_2D_3$ in concentrations of 10^{-7} or 10^{-6} M for 150 hr was consistently associated with reductions in final population density (to 64 and 50% of control, respectively) when cultures were exposed to $1,25\text{-}(OH)_2D_3$ beginning in the early exponential period of growth. Growth suppression as a function of $1,25\text{-}(OH)_2D_3$ was observed only in DM; addition of the vitamin to cells cultured in SSM had no effect on growth properties. The reduced population density seen in DM could not merely be attributed to $1,25\text{-}(OH)_2D_3$ associated cytotoxicity since cell viability (as determined by Trypan Blue dye exclusion assay at 24, 48, 96 and 150 hr of exposure) was unchanged (approximating 96 ± 2%) throughout the treatment period. The doubling times of cells grown in medium containing 10^{-7} or 10^{-6} M $1,25\text{-}(OH)_2D_3$ were significantly longer (a mean increase of 12 and 15 hr respectively) compared to untreated populations propagated in DM; at a concentration of 10^{-8} M, $1,25\text{-}(OH)_2D_3$ had no discernible effect on cellular growth kinetics (Table 3). Population doubling times of control cells were not significantly different from dexamethasone (10^{-7} M)-treated cells (mean = 32.1 and 33.9 hr respectively), indicating that growth restriction was apparently not merely a non-specific steroid effect.

Growth restriction of 77-1/3a cells in response to $1,25\text{-}(OH)_2D_3$ was reflected in a reduction (relative to control, untreated, cells at equivalent population densities) in the mean cellular content of p35 (when assayed at 120 hr after replacement of the growth medium with DM containing 10^{-7} M $1,25\text{-}(OH)_2D_3$) (Table 4). The down-regulation in cellular proliferative rate and p35 content was not a permanent aspect of $1,25\text{-}(OH)_2D_3$ exposure since replacement of vitamin D-containing DM with non-supplemented DM was associated with restoration (within 24–48 hr) of normal growth rate and p35 content. In contrast, addition of $1,25\text{-}(OH)_2D_3$ did not alter the albumin secretory rate (expressed as micrograms of albumin secreted per ml; normalized per 10^6 cells over a 24-hr period) (Table 4) nor influence the number of albumin-secreting cells as determined by immunofluorescence microscopy (data not shown).

The decreased cellular p35 content and reduced proliferative rate seen in $1,25\text{-}(OH)_2D_3$-treated 77-1/3a cells were similar, both in trend and degree, to the decline in growth and p35 levels seen in contact inhibitory factor-treated rat hepatic tumor cells [20],

Table 2. Relative growth capacities of 77-1/3a cells as a function of DM growth supplements

Media	Population saturation density* (cells × 10^{-5})	% of DM†
DM	28.5 ± 4.1	100.0
DM − insulin	22.9 ± 3.7	80.4
DM − glucagon	19.8 ± 3.3‡	69.5
DM − linoleic acid: albumin complexes	26.6 ± 2.9	93.3
DM − epidermal growth factor	8.7 ± 1.3§	30.5

Equivalent numbers of 77-1/3a cells were seeded to tissue culture dishes containing SSM; after 24 hr, the medium was changed to DM or DM without the indicated supplements. Cells were propagated for 7 additional days in these media at which time the cultures were harvested by trypsinization and cells counted by hemocytometer.

* Values are means ± SD of triplicate determinations.

† Calculated using group mean.

‡§ Significantly different from DM: ‡$P < 0.05$, or §$P < 0.005$.

Table 3. Effect of 1,25-$(OH)_2D_3$ on growth of 77-1/3a cells in chemically defined medium

Concentration of 1,25-$(OH)_2D_3$ (M)	Cell number at day 7 (× 10^{-5})	Doubling time (hr)
0 (EtOH)	2.8 ± 0.3	33 ± 3
10^{-6}	1.4 ± 0.1*	48 ± 4†
10^{-7}	1.8 ± 0.2†	45 ± 3†
10^{-8}	2.4 ± 0.2	36 ± 4

77-1/3a Cells were cultured in SSM for 24 hr and switched to DM containing ethanol or various concentrations (10^{-6}–10^{-8} M) of 1,25-$(OH)_2D_3$ dissolved in EtOH (20 mM final concentration). At days 3, 4, 5, 6, and 7, cells in triplicate plates from each group were counted. The data represent mean ± SD. Population doubling time was calculated by regression analysis of culture density (cells per culture) as a function of time of exposure during the period of exponential phase growth.

*† Significantly different from untreated control: *$P < 0.005$, and †$P < 0.01$.

DMSO-differentiated Friend erythroleukemia cells [11], and retinoic acid-induced quiescent (but non-differentiated) Friend cells [21]. These data are consistent with the concept that cellular p35 content is altered (within a certain restricted range) as a consequence of proliferative perturbation, rather than differentiated status, of various tumor cell types [21, 22]. This is supported by the observations that: (a) 1,25-$(OH)_2D_3$ did not alter the pattern of albumin secretion in 77-1/3a cells, whereas differentiating-inducing agents do alter this secretory profile (e.g. [10, 12, 18]); (b) actively proliferating fetal liver cells express relatively high levels of p35 compared to the quiescent adult organ [23]; and (c) release of Friend cells from the retinoid-induced quiescent G_{1T} substrate [24] is accompanied by a rapid increase in p35 expansion [21]. It remains to be determined whether 77-1/3a cells are prolonged in any particular cell cycle phase as a consequence of 1,25-$(OH)_2D_3$ exposure. Recent data [21], however, suggest that (at least for retinoid-induced erythroleukemia cells)

Table 4. Effect of 1,25-$(OH)_2D_3$ on cellular p35 content and albumin secretion

Growth conditions	Albumin secretion ($\mu g/10^6$ cells × 24 hr)	p35 Content (% control)
Control (EtOH)	13.9 ± 1.3	100.0
1,25-$(OH)_2D_3$ (10^{-7} M)	14.1 ± 2.0	61.0 ± 6.6
1,25-$(OH)_2D_3$/control	ND	97.1 ± 2.8

77-1/3a Cells were cultured in SSM for 24 hr and then in DM. Cells were analyzed for cellular p35 content, and the medium was analyzed for albumin content at 120 hr after change-over to DM + 20 mM EtOH (control) or DM + 10^{-7} M 1,25-$(OH)_2D_3$ (for quantitation of albumin secretory rate, the medium was changed 24 hr prior to harvest). In one group of cultures exposed to 1,25-$(OH)_2D_3$, the medium was changed after 120 hr of vitamin D treatment to control medium (EtOH only), and the cellular p35 content was determined 48 hr after 1,25-$(OH)_2D_3$ recovery. For estimation of p35 content, the mean value for control cells was 477 densitometric units/mg of protein assayed and 291 and 462 units for 10^{-7} M 1,25-$(OH)_2D_3$ and recovered cells respectively. Data represent mean ± SD of at least triplicate immunoblot determinations (Materials and Methods). ND: not determined.

decreased p35 content is a programmed aspect of cell quiescence and independent of cell cycle substrate.

Clearly, 1,25-$(OH)_2D_3$ produces similar growth restriction in other epithelial (e.g. colon) tumor cells [25], although in these cases the tissue is presumed to contain the appropriate 1,25-$(OH)_2D_3$ receptor. Whether the restricted proliferative response of epithelial tumor cells derived from a supposed non-target tissue (such as liver) is similarly mediated by a "receptor" remains to be determined. It would be relevant to compare the growth response of 77-1/3a cells with other, particularly more highly-differentiated, tumor cells of hepatic origin since liver itself derives from primitive gut endoderm.

The circulating level of 1,25-$(OH)_2D_3$ in a normal individual is $\approx 10^{-10}$ M, whereas the 1,25-$(OH)_2D_3$ concentrations required for induction of differentiation of the leukemia cells and inhibition of cell growth of other cancer cells are 10^{-8}–10^{-5} M [8, 26, 27]. In the present study, effects of 1,25-$(OH)_2D_3$ were observed at a concentration of $\geq 10^{-7}$ M. It is unknown why a pharmacological dose of 1,25-$(OH)_2D_3$ is required for the induction of a tumor cell response. The requirement of a pharmacological dose is similar to the phenomenon that a high dose of retinoic acid (3×10^{-3} M) is necessary to inhibit human rectal adenocarcinoma cell growth in culture [28], in spite of the fact that retinoic acid is required for the normal differentiation of epithelial tissues [29].

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