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1,25-Dihydroxyvitamin D_3, but not retinoic acid, induces the differentiation of U937 cells

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SUMMARY

We have examined the effects of vitamin D_3 metabolites and retinoic acid on the myelomonocyte cell line U937. Inhibition of proliferation, measured by incorporation of ^{125}iodo-deoxyuridine was seen at 72 h with 1,25-$(OH)_2D_3$ but not 25$(OH)D_3$ or 24, 25$(OH)_2D_3$ metabolites. CD14 molecules, not normally present on U937 cells, were induced on the cell surface. However, Class II major histocompatibility complex (MHC) molecules were not induced and Class I MHC molecules not increased in density as determined by flow cytometry. Retinoic acid inhibited proliferation but failed to induce CD14 molecules. These data suggest that both 1,25$(OH)_2D_3$ and retinoic acid act as an anti-proliferation signal to U937 cells; only 1,25-$(OH)_2D_3$ induces the differentiation towards a more mature phenotype.

Keywords cell differentiation retinoic acid 1,25-dihydroxyvitamins D_3

INTRODUCTION

The vitamin D endocrine system has recently been shown to influence immune responses. Receptors for the active metabolite of vitamin D_3, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), are present in monocytes and activated T cells, as well as in myeloid cell lines such as U937 (Peacock *et al.*, 1982; Bhalla *et al.*, 1983). The hormone, 1,25-$(OH)_2D_3$, inhibits proliferation of activated T cells by decreasing interleukin 2 production (Bhalla *et al.*, 1984; Rigby, Stacy & Fanger, 1984; Tsoukas, Provedinni & Manolagas, 1984), and it augments interleukin 1 production by macrophages (Bhalla, Amento & Krane 1986) and U937 cells (Amento *et al.*, 1984).

1,25-$(OH)_2D_3$ induces the differentiation of myeloid cells, such as murine M1 and human HL60, towards the monocyte lineage (Abe *et al.*, 1981, Bar-Shavit *et al.*, 1983). Retinoids also have powerful influences on cell proliferation and differentiation (Sporn & Roberts, 1983; Goodman, 1984). Retinoic acid can trigger the differentiation of HL-60 cells towards granulocytes probably by interacting with a specific cytoplasmic retinoic acid binding protein (Breitman, Selonic & Collins, 1980). We have examined the effects of vitamin D_3 metabolites and retinoic acid on the induction of differentiation of U937 cells. We found that 1,25-$(OH)_2D_3$, but not other vitamin D_3 metabolites, inhibited the proliferation of U937 cells and induced expression of CD14, a monocyte marker, suggesting differentiation of the cells along the monocyte pathway. In contrast, retinoic acid inhibited cell proliferation but failed to induce CD14.

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MATERIALS AND METHODS

Reagents

The metabolites 1,25 dihydroxyvitamin D_3, (1,25-$(OH)_2D_3$) 24,25dihydroxyvitamin D_3 (24,25$(OH)_2D_3$) and 25 hydroxyvitamin D_3 (25-$(OH)D_3$) were a kind gift from Dr Uskokovich, Hoffman-La Roche, Nutley, NJ. Each metabolite was dissolved in 100% ethanol. The final concentration of ethanol in culture did not exceed 0·1%. Retinoic acid was obtained from Sigma Chemical Company and dissolved in 100% ethanol. RPMI1640 tissue culture medium and fetal calf serum were obtained from Gibco.

Cells and culture conditions

U937 cells were maintained in RPMI 1640 tissue culture medium supplemented with 5% fetal calf serum, L-glutamine (2mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells in log phase were incubated at 2×10^5/ml for up to 72 h in the presence of 10^{-9}–10^{-7}M of each metabolite of vitamin D_3 or 10^{-7}M of retinoic acid. Controls were included containing the corresponding amount of ethanol, which had no effect on cell proliferation or differentiation. Cell viability was assessed by staining with ethidium bromide/acridine orange followed by fluorescence microscopy.

Measurement of cell proliferation

Control (ethanol only) and treated cells were incubated in microtitre well plates and pulsed with 0·5 μCi/well of ^{125}IUdR (Amersham) for the last 6 h of incubation. Cells were then

harvested using a cell harvester (Titertek) and the amount of incorporated radioactivity measured using a gamma counter. Data are expressed as counts per minute (ct/min).

Cell differentiation

The induction of the surface glycoprotein CD14 was used as a marker of cell differentiation. CD14 is expressed on mature monocytes/macrophages and is not expressed by U937 cells or monocytes in their early stages of differentiation (Todd *et al.*, 1982). UCHM1 monoclonal antibody (kindly supplied by Dr P. Beverley) was used to detect surface CD14 (Hogg *et al.*, 1984). Cells (5×10^5) were incubated on ice with 20 μl of a dilution of UCHM1 previously titrated to give plateau values on blood monocytes. Twenty microlitres of IgG Cohn Fraction II were added simultaneously to block binding through Fc receptors. Cells were also incubated with monoclonal antibodies directed against Class I (W632) and Class II MHC (Mid 3) (Lydyard *et al.*, 1985). An antibody isotype of irrelevant specificity (UCHT1, directed against CD3) was included as an additional control for Fc receptor binding. After 45 min incubation, cells were washed twice in 0·2% BSA in PBS and incubated for a further 30 min on ice with 30 μl of fluorescein-isothiocyanate-conjugated $F(ab)_2$ fragment of rabbit anti-mouse Ig (Dako) (1 : 15 dilution). Cells were then washed three times and fixed in 1% formaldehyde for analysis either on an EPICS C flow cytometer (Coulter Electronics, Luton, UK) or a FACSCAN flow cytometer (Becton Dickinson, CA). Log amplification was used for analysis.

RESULTS

Effects of vitamin D metabolites on cell proliferation

Whilst 1,25-$(OH)_2D_3$ inhibited proliferation of U937 cells in a dose-dependent manner, no inhibition was observed when 25-$(OH)D_3$ or 24,25$(OH)_2D_3$ were used (Table 1). At 10^{-9}M, a concentration within the normal physiological levels of 1,25 $(OH)_2D_3$, the hormone inhibited proliferation by up to 78 ± 5% of control. In time-course studies, over 24–72 h, significant inhibition of cell proliferation was observed after 48 h of culture

Table 1. Effects of vitamin D_3 metabolites on proliferation of U937 cells

Metabolite	Concentration (M)	Experiment 1	Experiment 2
	—	46646 (3288)	47872 (2582)
1,25-$(OH)_2D_3$	10^{-9}	36451 (1910)*	—
	10^{-8}	33525 (3831)*	32092 (2472)*
	10^{-7}	30509 (1109)*	30076 (2948)*
25$(OH)D_3$	10^{-9}	46183 (1948)	—
	10^{-8}	46631 (2471)	—
	10^{-7}	45981 (2767)	—
24,25$(OH)_2D_3$	10^{-8}	—	47060 (2776)
	10^{-7}	—	47512 (2642)

U937 cells were cultured alone or with various concentrations of vitamin D_3 metabolites. Cell proliferation was determined at 72 h by the incorporation of ^{125}IUdR. Results are expressed as mean ct/min with standard error in parentheses.

* $P < 0.01$.

Table 2. Time course for the effect of 10^{-8}M 1,25$(OH)_2D_3$ on U937 cell proliferation

Experiment no.		Time (h) 24	48	72
1	Control	53705 (2282)	76296 (1357)	104155 (1999)
	1,25$(OH)_2D_3$	56120 (4724)	67432 (3325)* [88%]†	75980 (5544)* [79%]
2	Control	24947 (4037)	65110 (2922)	102123 (5383)
	1,25$(OH)_2D_3$	29399 (2105)	52242 (2463)* [80%]	68443 (1796)* [67%]
3	Control	16979 (2328)	42937 (2015)	55145 (6670)
	1,25$(OH)_2D_3$	12391 (2819)	30567 (2590)* [71%]	29441 (2261)* [53%]

Results are expressed as mean ct/min with standard error in parentheses.

U937 cells at 2×10^5/ml were incubated with or without 10^{-8}M 1,25$(OH)_2D_3$ for 24 to 72 h after which cell proliferation was assayed. Control cells were incubated with a corresponding dilution of carrier (ethanol).

* $P < 0.01$.

† Figures in square brackets are percentages of control values.

Table 3. Effect of 10^{-8}M 1,25$(OH)_2D_3$ on surface markers on U937 cells

Experiment no.	Marker	Time (h) 0	24	48	72
1	CD3	< 1 (0)	< 1 (0)	< 1 (0)	1 (0)
	CD14	1 (0)	75 (104)	81 (149)	69 (136)
	MHC I	100 (202)	99 (205)	99 (206)	100 (195)
2	CD3	2 (0)	2 (0)	2 (0)	1 (0)
	CD14	2 (0)	53 (95)	66 (80)	58 (93)
	MHC I	100 (183)	99 (214)	99 (195)	99 (182)
	MHC II	2 (0)	2 (0)	1 (0)	2 (0)
3	CD3	1 (0)	1 (0)	1 (0)	2 (0)
	CD14	1 (0)	50 (0)	66 (112)	55 (115)
	MHC I	100 (200)	100 (230)	100 (225)	99 (225)
	MHC II	3 (0)	—	—	4 (0)

U937 cells were cultured in the presence or absence of 10^{-8}M 1,25$(OH)_2D_3$ for up to 72 h. At 0, 24, 48, 72 h cells were assayed for the presence of cell surface markers CD3, CD14, MHC Class I and II. Results are expressed as percentage positive. The figures in parentheses are the mean fluorescence intensities (MFI).

(to 79 ± 8·5% of control) with 1,25$(OH)_2D_3$. After 72 h, proliferation had decreased further to 64 ± 10·3% of control values (Table 2). This was confirmed by measurement of cell numbers. After 72 h of culture the control, 1,25 $(OH)_2D_3$-treated and 25 $(OH)D_3$-treated cultures had expanded to $8{\cdot}8 \times 10^5$/ml, $3{\cdot}8 \times 10^5$/ml and $8{\cdot}2 \times 10^5$/ml, respectively.

Table 4. Effects of Retinoic acid on cell proliferation and surface antigen changes

	Cell proliferation*			Cell surface changes†			
	Experiment 1	Experiment 2	Experiment 3	Marker	Control	Retinoic acid	$1,25(OH)_2D_3$
Control	27271 (2554)	19181 (1188)	26668 (283)	CD3	1 (0)	1 (0)	<1 (0)
Retinoic acid	14594 (12631)‡	14226 (1476)‡	16026 (656)‡	CD14	<1 (0)	2 (0)	55 (144)
				MHC I	99 (245)	97 (210)	98 (200)

U937 cells were cultured for 72 h alone (with ethanol), with 10^{-7}M retinoic acid or with $1,25(OH)_2D_3$ at 10^{-8}M. Cell proliferation, from three experiments, in the presence or absence of retinoic acid is shown. Surface markers were analysed on each occasion and a representative result is shown.

* Results expressed as mean ct/min (s.e.m.).

† Results expressed as percentage positive cell (MFI).

‡ $P<0.01$.

Induction of differentiation by $1,25(OH)_2D_3$

U937 cells cultured in the presence of 10^{-8}M $1,25(OH)_2D_3$ were examined for the presence of CD14 and MHC Class I and II surface antigens (Table 3). The density of Class I molecules, detected by the monoclonal antibody W632, remained unaffected by the hormone, while expressions of CD14 was increased by it. An increase in surface CD14 was apparent 24 h after the addition of the hormone, when $59\pm13\%$ of cells stained positive. After 48 h, $71\pm9\%$ of cells exhibited the marker. A slight fall to $61\pm7\%$ positive cells was seen at 72 h. In four other experiments the peak percentage of positive cells was also detected at 48 h (data not shown). Class II MHC molecules were not induced, and $25\text{-}(OH)D_3$ did not induce CD14.

Comparisons with retinoids

Retinoic acid is a differentiating agent of myeloid cell lines such as HL-60 (Breitman *et al.*, 1980). After 72 h of culture, 10^{-7}M retinoic acid inhibited the proliferation of U937 cells by $62\pm11\%$, comparable with the effects of $1,25\ (OH)_2D_3$ (Table 4). Unlike $1,25(OH)_2D_3$, retinoic acid failed to induce the expression of CD14 (Table 4).

DISCUSSION

We have shown that the physiologically active metabolite of vitamin D_3, $1,25\text{-}(OH)_2D_3$, specifically inhibits the proliferation of U937 cells whilst inducing the monocyte-related surface antigen CD14. The failure of other metabolites of vitamin D_3 to induce these changes suggests that the hormone $1,25(OH)_2D_3$ mediates these events by high affinity binding to its intracellular receptor. This is consistent with the data of others (Abe *et al.*, 1981; Amento *et al.*, 1984). Furthermore, this interpretation is supported by our observation that in other myeloid cell lines, KG1 and KG1a, lacking the $1,25\text{-}(OH)_2D_3$ receptor, the hormone fails to inhibit proliferation (unpublished observations).

The inhibition of cell proliferation was maximal after 72 h exposure to $1,25\text{-}(OH)_2D_3$. The induction of CD14 was an earlier event detectable at 24 h, which peaked at 48 h and was slightly, but not significantly, reduced at 72 h. This reduction may be a consequence of a change in the effective concentration of the hormone due to its utilization by the cells or its catabolism. However, since cell proliferation continues to decline at 72 h this is unlikely to be the only explanation. It is also possible that a subset of U937 cells are resistant to the action of $1,25\text{-}(OH)_2D_3$ and continue to proliferate and that by 72h they form a significant proportion of the total number of cells present in culture. Further work is in progress to 'grow out' a subset of U937 cells resistant to the action of $1,25\text{-}(OH)_2D_3$.

The induction of CD14 antigen is consistent with the suggestion that $1,25\text{-}(OH)_2D_3$ promotes the differentiation of myeloid cell lines toward more mature monocytes (Bar-Shavit *et al.*, 1980; Miyaura *et al.*, 1981; Amento *et al.*, 1984). However, the effects of the hormone on MHC Class I and Class II expression varies depending on the stage of differentiation of myeloid cell lines and the cell type. U937 cells constitutively express MHC Class I antigen which is unaffected by $1,25(OH)_2D_3$, whereas in HL-60 cells Class I expression is enhanced by the hormone (Ball *et al.*, 1984). Class II surface antigens are not inducible on U937 but monocytic differentiation of Hl-60 by $1,25(OH)_2D_3$ is accompanied by induction of MHC Class II (Rossi *et al.*, 1987). In the murine myelomonocytic line, WEHI-3, $1,25(OH)_2D_3$ augments MHC Class II expression previously induced by interferon-gamma (Morel *et al.*, 1986). In contrast, $1,25(OH)_2D_3$ down-regulates Class II expression in cells which constitutively express these molecules, such as peripheral blood monocytes/macrophages (Amento *et al.*, 1986) and the melanoma cell line DUMe117 (Carrington *et al.*, 1988).

Further differences in responses of myeloid cell lines to different agents are shown by our results with retinoic acid which inhibited the proliferation of U937 cells but failed to induce CD14. In contrast, retinoic acid inhibits the proliferation of HL-60 and induces granulocytic differentiation (Breitman *et al.*, 1980). This suggests that the anti-proliferative effects and differentiation are dissociable events. This potential to differentiate into granulocytes is not present in U937 cells which are arrested at a later stage of myeloid differentiation than HL-60 (Koffler, 1983). Although it has been reported that retinoic acid induces some differentiation of U937 cells, this was on the basis of their ability to reduce nitroblue tetrazolium (a measure of destructive enzyme activity in the cells) and surface antigens were not examined (Olsson *et al.*, 1983). The induction of this activity in U937 cells might reflect the action of retinoic acid as an 'activator'. This might be similar to the $1,25(OH)_2D_3$-mediated enhancement of the production of interleukin 1 by

monocytes (Bhalla *et al.*, 1986). Retinoic acid did not affect MHC Class I or induce MHC Class II.

Although the behaviour of cell lines may differ from that of normal cells, the ability of $1,25(OH)_2D_3$ to induce differentiation of myeloid cell lines towards monocytes suggests that the hormone may have a physiological role in the development of myeloid cells in the bone marrow. This may be achieved by the delivery of the hormone by the vasculature, or more likely through its local production by macrophages which have the capacity to synthesize $1,25(OH)_2D_3$ from its precursor $25(OH)D_3$ (Adams *et al.*, 1983; Mason *et al.*, 1984). Furthermore, $1,25(OH)_2D_3$ may affect the function of more mature myeloid cells (monocytes/macrophages) by enhancing cytokine production and modulating antigen presentation function. Retinoic acid, on the other hand, could operate earlier in the differentiation of the myeloid cells and 'drive' the differentiation towards granulocytes, as well as inhibiting the growth of more mature myeloid cells. Further experiments are in progress aimed at dissecting more clearly the mechanism responsible for cell differentiation by $1,25(OH)_2D_3$. It is hoped that this will provide a clearer understanding of the role of this hormone in myeloid differentiation in the bone marrow and on mature forms of the lineage.

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