Differentiation of a Human Monocytic Cell Line by 1,25-Dihydroxyvitamin D₃ (Calcitriol): A Morphologic, Phenotypic, and Functional Analysis

William F.C. Rigby, Li Shen, Edward D. Ball, Paul M. Guyre, and Michael W. Fanger

Several recent studies have suggested a role for 1,25-dihydroxyvitamin D₃ (calcitriol) in myeloid differentiation. We have examined the effects of calcitriol on the U937 monoblast cell line and found that calcitriol, at near physiologic concentrations, is a potent inhibitor of U937 growth. Moreover, calcitriol induces differentiation to a monocyte-macrophage phenotype marked by enhanced α-naphthyl esterase staining. Morphologic changes were attended by de novo induction of the myeloid specific antigen detected by the monoclonal antibody AML-2-23, as well as dramatic increases in Fc receptors for IgG. In addition, calcitriol induced U937 cells to perform phagocytosis and antibody-dependent cellular cytotoxicity. These results indicate the potential activity of calcitriol in myeloid differentiation and additionally suggest a role for calcitriol in monocyte-macrophage activation.

MATERIALS AND METHODS

Cells

The U937 cell line¹ was provided by Dr P. Ralph, Memorial Sloan-Kettering Institute (New York). Cells were continuously cultured in RPMI 1640 (K.C. Biologicals, St Louis) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sterile System Inc, Logan, Utah) and gentamicin (50 μg/mL, Schering Corp, Kenilworth, N.J). The same lot of FBS was used in all experiments.

Vitamin D Compounds

Preservative free 1,25-dihydroxycholecalciferol (calcitriol) was the generous gift of Dr Milan R. Uskokovic, Hoffman-LaRoche Inc, Nutley, NJ. 25-Hydroxycholecalciferol (25OHDS) was kindly provided by Dr Rodney Carlson, Upjohn Co, Kalamazoo, Mich. Both compounds were dissolved in 95% sterile ethanol and stored in glass vials at −20°C.

Cell Cultures

U937 cells in log phase growth were seeded in tissue culture flasks (Becton Dickinson, Oxnard, Calif) at 10⁶ cells/mL. Calcitriol and 25OHDS, dissolved in 95% ethanol, were added, with final ethanol concentrations always less than 0.1%. Control cultures included the same concentrations of ethanol. Cells were cultured for 96 hours at 37°C in a 5% CO₂ humidified atmosphere and then removed for the various studies.

Morphology

Cells were assessed for morphologic change by comparisons of Wright-Giemsa-stained cytospin preparations after 96 hours of culture. Increased expression of cytoplasmic α-naphthyl acetate esterase (nonspecific esterase) was determined by counting the percentage of cells with maximal staining compared with background.¹⁴

Growth Inhibition

U937 cells were suspended at 5 x 10⁴ cells/mL in media supplemented with calcitriol (10⁻⁶ to 10⁻⁸ mol/L) and incubated at 37°C. On day 3, cells were suspended, and 100-μL volumes of each culture were removed and plated in triplicate into 96-well plates (Becton Dickinson). Cultures were pulsed with 0.5 μCi of tritiated thymidine ('H-Thy) (70 Ci/mmol, New England Nuclear, Boston) for two hours at 37°C, and harvested with a Mash II Cell Harvester (Microbiological Associates, Walkersville, Md) onto glass microfiber filter paper (Thomas Scientific, Philadelphia). After drying, filters were added to tubes containing 4 mL Aquasol-2 (New...
after five days of culture, cultures were resuspended with the number and percentage of viable cells determined by ethidium bromide/acridine orange staining. Cell viability in both control and treated cells were similar (>90%). Results using 3H-Thy are represented as the mean of triplicate cultures. Inhibition of growth by calcitriol was calculated by the equation: % Inhibition = 1 – (cell count or cpm calcitriol culture)/(cell count or cpm control culture).

Monoclonal Antibodies

The reactivity of control and treated cells with various monoclonal antibodies (MoAbs) was examined by flow cytometry. The antibodies used included AML-2-23, a myeloid specific antibody of the IgG2 class that reacts strongly with monocytes and weakly with granulocytes; 62D2, an IgG2 antibody that reacts with a constant epitope on the heavy chain of class I HLA antigens (BRL, Gaithersburg, Md); OKIa1 (Ortho Diagnostics, Raritan, NJ), an IgG2 antibody that reacts with the Fe receptor for IgG class that reacts strongly with monocytes and weakly with IgG2b monoclonal human IgGI as previously described. Cell aliquots (106) containing 1 mg/mL bovine serum albumin (BSA) and 0.5 mg/mL sodium azide (Fisher Scientific, Fairlawn, NJ) at 4 °C, then incubated for 30 minutes at 4 °C with fluorescent isothiocyanate (FITC) conjugated goat F(ab')2 antibody directed to mouse antibody (Boehringer-Mannheim, Indianapolis). Detection of IgGl, FcR bearing cells was performed using FITC-conjugated monoclonal human IgG1 as previously described. Cell aliquots (106) were mixed with an equal volume of 10−5 mol/L FITC-labeled monoclonal human IgG1 (IgGl-FITC) and incubated at 37 °C for two hours in the absence or presence of 10−4 mol/L identical unlabelled monoclonal IgG1 (to assess nonsaturable binding). Under these conditions there is less than 10% internalization of cell-associated ligand. All samples were washed and resuspended in 0.5 mL of PBS containing 1 mg/mL BSA and 0.5 mg/mL sodium azide and 106 cells analyzed by cytofluorography.

Complement and Fc Receptor Studies

Antibody-coated bovine erythrocytes (BBRC; Wilfer Laboratory, Stillwater, Minn) were prepared with subagglutinating amounts of IgM (E-IgM) and IgG (E-IgG) antibodies as previously described. Rosettes were formed by mixing equal volumes of U937 cells (2×106 cells/mL) with 1% sensitized erythrocytes. The mixtures were centrifuged at 200 g, incubated on ice for 30 minutes, the pellet gently resuspended in a solution of acridine orange and ethidium bromide, and viable rosette-forming cells (>3 RBC per cell) were counted, using UV and incident light.

Phagocytosis

Bovine erythrocytes were coated with various concentrations of IgG as described above. Phagocytosis was evaluated by incubating control and calcitriol-treated U937 cells with erythrocytes (E) or antibody-coated erythrocytes (E-IgG, E-IgM) for 30 to 60 minutes at a target-effector ratio of 5:1. Noninternalized erythrocytes were then lysed with buffered ammonium chloride. The U937 cells were cytocrifuged onto slides stained with Wright-Giemsa, and the percentage of phagocytosis determined by counting 300 cells.

Antibody-Dependent Cell Cytotoxicity

Antibody-dependent cell cytotoxicity (ADCC) was performed as previously described. Briefly, freshly drawn chicken erythrocytes (CE) were washed in RPMI 1640, incubated with 200 µCi of 51Cr-sodium chromate (New England Nuclear) for 1½ hours at 37 °C, washed, and resuspended at 2×106 cells/mL in RPMI 1640 containing 10% FBS. Equal volumes (50 µL) of labeled CE targets, either control or treated U937 effector cells, and anti-CE IgG at the concentrations indicated were mixed in round-bottomed microtiter wells (A/S Nunc, Denmark) and incubated for four hours, after which half the supernate was removed for determination of 51Cr release. Maximal lysis was achieved by addition of 100 µL of 2% detergent (“Count Off,” New England Nuclear). The percentage of cytotoxicity was determined according to the equation: % Cytotoxicity = (release with Ab – release without Ab)/(maximum lysis – spontaneous release).

RESULTS

Calcitriol effected a dose-dependent inhibition of U937 cell growth as determined by cell count after five days in culture (Table 1). Growth inhibition was first evident at 10−10 mol/L calcitriol, and near-maximal at 10−6 to 10−7 mol/L. The specificity of this growth inhibition and its relationship to vitamin D receptor binding were compared using calcitriol and its immediate biologic precursor, 25OHD3. Using incorporation of 3H-Thy as an index of DNA synthesis, it was found that calcitriol inhibited 3H-Thy incorporation by U937 cells in a concentration-dependent fashion (Fig 1). Comparable concentrations of 25OHD3, to which the vitamin D receptor binds ten- to a hundredfold less avidly,12 had little effect on growth. Higher concentrations (10−5 to 10−6 mol/L) of 25OHD3, resulted in

<table>
<thead>
<tr>
<th>Table 1. Growth Inhibition by Calcitriol*</th>
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<tbody>
<tr>
<td>Calcitriol Concentration (mol/L)</td>
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<tr>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>10−10</td>
</tr>
<tr>
<td>10−9</td>
</tr>
<tr>
<td>10−8</td>
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<td>10−7</td>
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*U937 cells were seeded at 5×105/mL and incubated for five days in various concentrations of calcitriol. Viable cells (>90% in control and treated cultures) were counted on day 5 using acridine orange and ethidium bromide staining and reported as the percentage of inhibition of cell growth. Inhibition of growth was calculated by the equation: % Inhibition = 1 – (cell concentration calcitriol culture)/(cell concentration control culture).
Inhibition of $^3$H-thymidine incorporation by U937 cells after three days in culture with different concentrations of calcitriol (1,25(OH)$_2$D$_3$) (△) and 25-hydroxyvitamin D$_3$ (25OHD$_3$) (□). Aliquots of cells (100 μL) were plated in triplicate in 96-well plates and pulsed with 0.5 μCi/well for two hours, harvested, and counted. Error bars denote standard deviation of triplicate samples. Percentage of inhibition was calculated as % Inhibition = 1 - (cpm calcitriol culture)/(cpm control culture).

**Morphology**

The inhibition of U937 growth by calcitriol (10$^{-8}$ mol/L) was accompanied by striking morphologic changes consistent with the induction of a monocyte-macrophage phenotype. After four days in culture, Wright-Giemsa staining of cytospin cell preparations revealed increased cell size with a decrease in the nuclear-cytoplasmic ratio and loss of cytoplasmic basophilia. In addition, calcitriol-induced cells developed pseudopodia, ruffling of their plasma membrane, adhered to plastic, and spread on glass surfaces. There were no multinucleated cells in either treated or control cultures. These changes were partially demonstrable at calcitriol concentrations of 10$^{-10}$ mol/L, and at 10$^{-8}$ mol/L were apparent in 100% of cells. Furthermore, the percentage of cells with maximal nonspecific esterase staining increased from 8% in controls to 81% in calcitriol-treated (10$^{-8}$ mol/L) cells. Similar morphologic changes were induced by 25OHD$_3$ only at very high concentrations (10$^{-5}$ to 10$^{-6}$ mol/L).

**Monoclonal Antibody Studies**

Cell surface antigen modulation on U937 cells by calcitriol was evaluated using MoAbs and cytofluorography (Table 2). The percentage of U937 cells positive for AML-2-23 increased from 5% to 77% as a result of culture with calcitriol (10$^{-8}$ mol/L). In addition, there was a greater than 15-fold increase in the mean fluorescent intensity (MFI) of AML-2-23 staining of all U937 cells (Fig 2, top). Other studies revealed that calcitriol induced almost a 100% increase in the expression of class I HLA antigens. However, no change in HLA-DR associated antigens detected by the MoAb OKIa1 was apparent. Last, there was no significant change in PM-81 binding after calcitriol treatment.

Calcitriol also induced marked increases in the FcR for IgG1 (Table 2 and Fig 2, bottom). The percentage of cells expressing detectable FcR for IgG1 increased more than twofold, with a nearly fourfold increase in

### Table 2. Expression of Cell Surface Markers on U937 Cells After Treatment With Calcitriol

<table>
<thead>
<tr>
<th></th>
<th>AML-2-23</th>
<th>HLA</th>
<th>OKIa1</th>
<th>PM-81</th>
<th>IgG1-FcR</th>
<th>3G8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5 (13)</td>
<td>85 (176)</td>
<td>0.7 (24)</td>
<td>83 (252)</td>
<td>31 (48)</td>
<td>1 (89)</td>
</tr>
<tr>
<td>Calcitriol (10$^{-8}$ mol/L)</td>
<td>77 (229)</td>
<td>93 (300)</td>
<td>1.7 (11)</td>
<td>85 (254)</td>
<td>79 (177)</td>
<td>1 (95)</td>
</tr>
</tbody>
</table>

*U937 cells (10$^5$/mL) in RPMI 1640 with 10% FBS were incubated with or without calcitriol for four days, then assayed for antigen or Fc receptor expression using cytofluorography. Irrelevant IgG2b and IgM antibodies were used as negative controls. Value in parentheses denotes mean fluorescent intensity (MFI) as measured by cytofluorography of positive cells. The voltage setting of fluorescent photomultiplier tube was 950 V in this experiment.
MFI. Binding was completely blocked by an excess of identical unlabeled IgG1, indicating that it was specifically FcR mediated. In contrast, calcitriol treatment did not increase the binding of the MoAb 3G8, which detects the FcR for IgG expressed by neutrophils.

The effects of calcitriol on AML-2-23 and the FcR for IgG1 were concentration dependent (Fig 3). The expression of both FcR for IgG1 and the AML-2-23 antigen increased in a linear fashion with increasing concentrations of calcitriol. Expression of FcR for IgG1 was maximal at 10^{-7} mol/L calcitriol, and of AML-2-23 antigen at 10^{-5} mol/L calcitriol, the induction of AML-2-23 peaked at two days, while induction of FcR for IgG1 and inhibition of ^3H-Thy incorporation became maximal after three to four days (Fig 4).

**Rosette Formation and Phagocytosis Studies**

U937 cells were studied in both their native and induced state for their ability to form rosettes with BRBC alone (E), or BRBC coated with IgG (E-IgG) or IgM (E-IgM). After four days in culture with calcitriol (10^{-8} mol/L), the percentage of U937 cells forming rosettes with E-IgG had increased from 39% to 67% (Table 3). There was no detectable induction of IgM-FcR. Moreover, whereas untreated U937 cells demonstrated no detectable phagocytosis of sensitized BRBC, calcitriol induced a significant phagocytic capability in these cells (Table 3). In this representative experiment, the capacity to phagocytose was found in approximately half (49%) of the calcitriol-treated cells.

**Antibody Dependent Cellular Cytotoxicity**

U937 cells were studied for their ability to mediate lysis of ^51Cr-labeled CE in the presence of anti-CE IgG (Fig 5). At an effector-target (E-T) ratio of 10:1, uninduced U937 cells did not mediate ADCC. After induction with calcitriol, however, U937 cells expressed significant ADCC even at E-T ratios of 1.25:1.

**DISCUSSION**

Inhibition of growth of U937 cells by calcitriol was dose dependent, the effects becoming apparent at low concentrations (10^{-7} mol/L), approaching physiologic levels (10^{-5} mol/L). This inhibition of U937 cell growth by calcitriol was accompanied by induction of a monocyte–macrophage phenotype with increased nonspecific esterase staining, increased expression of the FcR for IgG1, as well as class I HLA antigens, and by de novo expression of the monocyte–granulocyte antigen detected by the MoAb AML-2-23. Calcitriol treatment also induced U937 cells to perform phagocytosis and to mediate ADCC. Demonstration that calcitriol, the most biologically active metabolite of vitamin D, can induce such changes, suggests that it has a role in myeloid differentiation. Furthermore, the immediate biologic precursor of calcitriol, 25OHD₃, is without

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**Table 3. Effect of Calcitriol on Fc Receptor Expression and Phagocytosis by U937 Cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rosette Formation (%)</th>
<th>Phagocytosis (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>E-IgG</td>
<td>E-IgM</td>
</tr>
<tr>
<td>Control</td>
<td>0 ± 1</td>
<td>0</td>
</tr>
<tr>
<td>Calcitriol (10^{-8} mol/L)</td>
<td>0 ± 1</td>
<td>0</td>
</tr>
</tbody>
</table>

*U937 cells were incubated in calcitriol (10^{-8} mol/L) for 96 hours. Control and calcitriol-treated cells were mixed with bovine erythrocytes sensitized with or without antibody (E, E-IgG, E-IgM), pelleted, and counted in a solution of acridine orange and ethidium bromide. Viable rosette-forming cells (>3 RBCs per cell) were counted as a percentage of 100 cells. Phagocytosis was measured by incubating control and calcitriol-treated cells for 30 minutes with erythrocytes with and without antibody sensitization. Noninternalized erythrocytes were lysed with buffered ammonium chloride. Cytocentrifuge preparations were stained with Wright-Giemsa and the percentage of U937 cells with internalized E determined after counting 300 cells.
Fig 5. Antibody-dependent cell cytotoxicity of #Cr-labeled chicken erythrocytes mediated by U937 cells cultured for four days in the absence (O) or presence (A) of 10^{-8} mol/L calcitriol. Error bars denote SD of triplicate samples.

 comparable effect unless used at ten- to one-hundredfold higher concentrations (10^{-5} to 10^{-6} mol/L). Since the vitamin D receptor has a much greater affinity for calcitriol than for 25OHD_3, it suggests that the induction of U937 cells by calcitriol is receptor mediated.

 Increases in AML-2-23 expression have been reported with other differentiating agents. Induction of this antigen accompanies HL-60 differentiation induced by dimethylsulfoxide, cis-retinoic acid, gamma interferon, or calcitriol (unpublished data). The modulation of AML-2-23 on U937 cells undergoing apparent morphologic maturation to a macrophage phenotype further confirms its utility as a marker of myeloid differentiation. Calcitriol also induced class I HLA antigen expression, as has been reported with HL-60 cells. Furthermore, gamma interferon has been reported to increase expression of FcR for IgG I on monocytes as well as U937 and HL-60 cells and has been suggested as being important in macrophage activation. Our demonstration of induction of FcR for IgG I on U937 cells by calcitriol thus suggests that it too may have a role in macrophage activation.

 This potential role of calcitriol in macrophage activation was further suggested by functional studies. Uninduced U937 cells have been shown to express approximately 20,000 IgG1-FcR per cell. Nevertheless, these cells were unable to mediate phagocytosis or ADCC of IgG-sensitized erythrocytes. With calcitriol induction, U937 cells acquired a significant ability to mediate phagocytosis or ADCC of IgG-sensitized erythrocytes. With calcitriol induction, U937 cells acquired a significant ability to mediate phagocytosis or ADCC of IgG-sensitized erythrocytes.

 The potential significance of calcitriol to macrophage differentiation and function in vivo has been amply suggested by studies demonstrating impaired immunity and recurrent infections associated with vitamin D deficiency. Impaired phagocytic function and decreased mobility by leukocytes has been shown in vitamin D-deficient states in both human and murine systems. This defect in phagocytic function was reversed by in vitro culture of macrophages with calcitriol, an observation consistent with our findings with U937 cells. Calcitriol may thus exert multiple influences on cells of the monocyte lineage, enhancing both differentiation of precursor cells as well as effector functions of more mature cells.

 It has been reported recently that pulmonary alveolar macrophages from patients with sarcoidosis have 1-hydroxylase activity and can synthesize calcitriol from 25OHD_3. This suggests the intriguing possibility that calcitriol may be released by macrophages as a monokine with growth inhibitory properties. Activation of macrophages or their recruitment from precursor cells, as suggested by the effects of calcitriol on U937 cells, suggests additional means by which calcitriol could function as a monokine.

 The possibility that calcitriol may be a physiologic regulator of myeloid growth and differentiation may be relevant to calcium homeostasis, since calcitriol has been shown to induce bone resorption by HL-60 cells. Calcium mobilization from bone is mediated by osteoclasts, which are believed to arise from hematopoietic precursors. Calcitriol may induce myeloid precursors to preferentially mature into osteoclasts with a bone-resorptive capability in order to increase serum calcium. Thus, the discovery of the activity of vitamin D in myeloid cell differentiation may lead to the elucidation of other mechanisms by which calcium homeostasis is maintained.

REFERENCES

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Differentiation of a human monocytic cell line by 1,25-dihydroxyvitamin D3 (calcitriol): a morphologic, phenotypic, and functional analysis

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