The Metabolite 1α,25-Dihydroxyvitamin D₃ Enhances Lymphokine-Mediated Activation of the Oxidative Metabolism of the U937 Cell Line and Phosphorylation of a 48-kD Respiratory Burst–Associated Protein

By Rose G. Snipes, Kirk Ways, Christopher N. D'Amico, Geetha Sivam, T. Kenney Gray, and Myron S. Cohen

U937 cells respond to a variety of stimuli with increased differentiation as manifested by reduced growth, increased adherence, increased expression of several surface receptors, and increased capacity for phagocytosis and formation of reactive oxygen intermediates. In the present study the effects of lymphocyte conditioned media, recombinant interferon-γ (IFN-γ), and 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) on the ability to form reactive oxygen intermediates by U937 cells were measured by using the luminol-dependent luminescence (LDL) assay. Neither 1,25(OH)₂D₃ alone nor IFN-γ alone enhanced competence for phorbol myristate acetate–stimulated LDL. Cells were capable of moderate LDL after exposure to lymphocyte conditioned media, and this was enhanced by 1,25(OH)₂D₃ (10⁻⁸ mol/L) and other vitamin D metabolites at higher concentrations. This effect was not secondary to accelerated production of myeloperoxidase, which is important in the LDL assay. Enhanced phorbol myristate acetate–stimulated phosphorylation of a 48-kd substrate was observed in ³²P-labeled intact cells treated with 1,25(OH)₂D₃ alone or in combination with IFN-γ. Treatment of cells with IFN-γ or lymphocyte conditioned media did not alter phosphorylation. These results support the concept that 1,25(OH)₂D₃ plays a role in phagocyte differentiation and activation beyond the effects of lymphokines. Protein kinase C–mediated phosphorylation reactions may be necessary for the ability of U937 cells to reduce O₂ and required for maximal activity under some conditions of incubation.

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From the Departments of Medicine, Microbiology, and Immunology, University of North Carolina School of Medicine at Chapel Hill.

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Address reprint requests to Myron S. Cohen, MD, Associate Professor of Medicine, Microbiology and Immunology, 547 Clinical Sciences Bldg, UNC School of Medicine at Chapel Hill, Chapel Hill, NC 27514.

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Aliquots were frozen at 0°C until use. Lymphocyte conditioned media were used at concentrations of 10% to 20% (vol/vol); the lymphokine preparation was only reduced in concentration in preparations that affected cell viability. Controls included lymphocytes isolated under identical conditions with PHA added to the lymphocyte conditioned media after the three-day incubation.

Luminol-Dependent Luminescence

Measurements of chemiluminescence were made with an ATP spectrophotometer (Model 3000, SAl Technology Co, San Diego). Light intensity was monitored continuously by using a strip chart recorder (Fisher Recordall, Pittsburgh) as previously described. Luminous events were also observed in a one-minute intervals from the digital readout of the spectrophotometer. The sensitivity was the same in all experiments (9.4). The temperature of the counting chamber was 31°C. The cell suspension was pelleted, media discarded, and cells resuspended in Hanks’ balanced saline solution (HBSS) at a final concentration of 5 x 10^6 cells/mL. These cells remained in a 37°C water bath until use. Background counts were obtained by adding 1.0 mL of a 10^-4 mol/L solution of luminol (Sigma) prepared in 1% bovine serum albumin to 1.0 mL of HBSS. Resting cell luminescence was obtained by adding 1.0 mL luminol to 1.0 mL of the cell suspension. Counts were obtained over a 15-minute interval. PMA (100 ng/mL) was used as a stimulus. Counts were recorded at one-minute intervals until peak luminescence was reached. Samples were examined in duplicate.

Myeloperoxidase Assay

The myeloperoxidase activity of U937 cells was determined by the O-dianisidine reaction as previously described. 11

Endogenous Substrate Phosphorylation in Intact Cells

Substrate phosphorylation was determined as previously described. 12-13 After six days of exposure to the various agents, the cells were centrifuged and resuspended in Eagle’s minimal essential medium with Earle’s salt but without glutamine or phosphates (GIBCO) for 30 minutes. A total of 3 x 10^6 cells were incubated with ^32P, 0.25 mCi, for two hours, after which the cells were centrifuged and the supernatant was removed. After resuspension in fresh medium, the cells were exposed to vehicle (MeSO, 0.01%) or PMA (100 ng/mL) for ten minutes. The reaction was terminated by adding a one-third volume of a solution containing 9% (wt/vol) sodium dodecyl sulfate, 15% (wt/vol) glycerol, 30 mmol/L Tris-HCl, pH 7.8, 0.05% (wt/vol) bromophenol blue, and 6% vol/vol mercaptoethanol. The mixture was heated to 110°C for three minutes. The samples were subjected to one-day polyacrylamide gel electrophoresis (PAGE) as described by Laemmli. 15 The stacking and running gels were 3% and 10% acrylamide, respectively. After staining and destaining, the gels were treated with 1 mol/L potassium hydroxide for one hour at 56°C. This procedure allowed optimal visualization of PMA-dependent substrate phosphorylations. After treatment for one hour in the destaining solution, the gels were dried, and autoradiography was performed. Concurrent electrophoresis of molecular weight (mol wt) standards allowed assignments of approximate mol wts to the phosphorylated substrates. Incorporation of ^32P into endogenous substrates was quantitated by densitometric analysis of the autoradiograms. The autoradiograms were scanned at 600 nm with a Gilford spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH).

RESULTS

Effects of 1,25(OH)2D3 and Lymphocyte Conditioned Media on the O2 Metabolism of U937 Cells

U937 cells were exposed to a variety of agents believed to be of importance in their differentiation. These included lymphokine conditioned media (harvested from cells stimulated with PHA), monoclonal IFN-γ, and 1,25(OH)2D3 and other vitamin D metabolites.

Table 1 demonstrates the effects on cells examined five days after incubation with lymphocyte conditioned media alone, 1,25(OH)2D3 alone, and 1,25(OH)2D3 plus lymphocyte conditioned media. Cells exposed to lymphocyte conditioned media alone demonstrated an increase in luminol-dependent luminescence (LDL) in response to PMA. Cells incubated with lymphocyte conditioned media plus 1,25(OH)2D3 showed a dramatic increase in LDL, whereas 1,25(OH)2D3 alone did not promote LDL.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Mean</th>
<th>SD</th>
<th>Increase Over Control (fold)</th>
<th>Number of Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>2</td>
<td>.50</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>.50</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>8.5</td>
<td>.50</td>
<td>4.2</td>
<td>14</td>
</tr>
<tr>
<td>CM + 1,25(OH)2D3 (10^-8 mol/L)</td>
<td>82</td>
<td>15.0</td>
<td>4.1</td>
<td>14</td>
</tr>
<tr>
<td>INF-γ, 1,000 U/mL</td>
<td>2</td>
<td>.50</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>INF-γ + 1,25(OH)2D3</td>
<td>45</td>
<td>12.0</td>
<td>22.5</td>
<td>14</td>
</tr>
<tr>
<td>CM + 24,25(OH)2D3 (10^-4 mol/L)</td>
<td>25</td>
<td>.50</td>
<td>12.5</td>
<td>3</td>
</tr>
<tr>
<td>CM + 25(OH)2D3 (10^-4 mol/L)</td>
<td>27</td>
<td>.50</td>
<td>13.5</td>
<td>3</td>
</tr>
<tr>
<td>1,25(OH)2D3</td>
<td>2</td>
<td>.50</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>24,25(OH)2D3</td>
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</tr>
<tr>
<td>25(OH)D3</td>
<td>2</td>
<td>.50</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>CM + 1,25(OH)2D3 + SOD</td>
<td>2</td>
<td>.20</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>CM + 1,25(OH)2D3 + azide</td>
<td>18</td>
<td>.45</td>
<td>9</td>
<td>3</td>
</tr>
</tbody>
</table>

The effects of incubation of U937 cells on competence for LDL stimulated by PMA (100 ng/mL). The cells were in culture for five days before the experiments were conducted. Background refers to emission of light by media. The control for these experiments was conditioned media harvested from lymphocytes not exposed to PHA. SOD was used at a concentration of 300 U/mL. One-millimolar azide was used.

Abbreviation: CM, lymphocyte conditioned media, 20% vol/vol.
Effects of IFN-γ and 1,25(OH)₂D₃ on O₂ Metabolism of U937 Cells

IFN-γ is a lymphokine believed critical to the differentiation and activation of monocytic phagocytes. The effects of IFN-γ were compared with lymphocyte condition media, as shown in Table 1. LDL was not observed among cells treated with INF-γ alone. Treatment of cells with INF-γ plus 1,25(OH)₂D₃ for five days enhanced competence for LDL but of less magnitude than cells treated with lymphocyte conditioned media and 1,25(OH)₂D₃ (Table 1).

To determine whether INF-γ resulted in the same degree of U937 cell differentiation after varied periods of incubation, the same comparisons were made among cells incubated for zero to seven days (Fig 1). At each time point the effects observed with lymphocyte conditioned media plus 1,25(OH)₂D₃ were equivalent to or greater than the combination of 1,25(OH)₂D₃ and IFN-γ.

Effect of Other Vitamin D Metabolites on the O₂ Metabolism of U937 Cells

The vitamin D metabolite most important for calcium homeostasis is 1,25(OH)₂D₃. Receptors for 1,25(OH)₂D₃ have been demonstrated in human monocytes. The metabolites 24,25(OH)₂D₃ and 25(OH)₂D₃ generally have lower affinity for such receptors than the active metabolite 1,25(OH)₂D₃. The effects of 1,25(OH)₂D₃ were compared with other metabolites of vitamin D (Table 1). Neither 24,25(OH)₂D₃ nor 25(OH)₂D₃ promoted maximal competence for LDL, even when concentrations as high as 10⁻⁴ mol/L were used.

Effect of Adherence of U937 Cells on LDL

Lymphokine plus 1,25(OH)₂D₃ increases U937 cell adherence from 3% to 57%. To determine the role of adherence in LDL, adherent cells were removed with a rubber policeman, and PMA-stimulated LDL was measured relative to an equivalent number of cells in suspension. As shown in Table 2, LDL was greater among adherent cells than cells in suspension, which suggests that competence for LDL is a parameter of differentiation correlated with adherence.

Mechanism(s) of Oxygen Reduction by U937 Cells

LDL results from the interaction of luminol with several reactive oxygen intermediates. As shown in Table 1, all light emission was eliminated by the addition of SOD to PMA-stimulated cells, thus indicating that superoxide was a critical species for this reaction. Light emission was inhibited to a lesser extent by sodium azide, which implied that a proportion of light could be attributed to the formation of hypochlorous acid, as has been reported for human neutrophils.

All U937 cells examined after differentiation expressed myeloperoxidase in equivalent concentrations (data not shown). These results suggest that differences in LDL related directly to the ability to form superoxide anion.

Phosphorylation of Endogenous U937 Substrates

Several recent studies have focused on protein kinase C activation and substrate phosphorylation during PMA-stimulated formation of reactive oxygen intermediates. Specifically, PMA-stimulated phosphorylation of a substrate with an approximate mol wt of 43 to 48 kD has been associated with superoxide formation, which suggests that these events are important in signal transduction. We examined the effects of 1,25(OH)₂D₃ on phosphorylation reactions in intact cells stimulated with PMA. U937 cells incubated with 10⁻⁴ mol/L 1,25(OH)₂D₃ for six days were labeled with ³²P and treated with PMA under conditions identical to those used to measure competence for LDL (Fig 2); longer exposure to PMA did not change these results (data not shown). When compared with control cells, 1,25(OH)₂D₃ exposure increased PMA-dependent phosphorylation of 68- and 48-kD substrates (Fig 2). PMA-dependent phosphorylation of a 17-kD substrate was not altered by 1,25(OH)₂D₃ pretreatment. Exposure to 1,25(OH)₂D₃ also enhanced the non-PMA-dependent phosphorylation of a 74-kD substrate. We also examined phosphorylation in cells treated with IFN-γ, lymphocyte conditioned media, or 1,25(OH)₂D₃ in the presence and absence of

Table 2. Peak LDL (cpm x 10⁶)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Adherent Cells</th>
<th>Nonadherent Cells</th>
<th>Percentage of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>104</td>
<td>54</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>124</td>
<td>60</td>
<td>48</td>
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<tr>
<td>3</td>
<td>84</td>
<td>64</td>
<td>76</td>
</tr>
<tr>
<td>4</td>
<td>75</td>
<td>50</td>
<td>66</td>
</tr>
<tr>
<td>Mean</td>
<td>93</td>
<td>57</td>
<td>60.5</td>
</tr>
</tbody>
</table>

Cells were cultured for five days in the presence of (20%) lymphocyte conditioned media and 1,25(OH)₂D₃ (10⁻⁴ mol/L), after which time nonadherent cells were prepared by gentle removal with a rubber policeman, adjusted for viability, and compared with adherent cells stimulated with PMA (100 ng/mL). Results from four separate experiments are given, and LDL of nonadherent cells is presented as a percentage of adherent cells.

Fig 1. Comparison of 1,25(OH)₂D₃ plus lymphocyte conditioned media or IFN-γ over seven days. Bars represent the mean and brackets the SEM of three to four separate experiments. The cells were stimulated with 100 ng/ml PMA.
IFN-γ (Table 3, Fig 3). PMA-stimulated, 48-kD substrate phosphorylation was detected under all conditions of incubation; cells exposed to lymphocyte conditioned media or IFN-γ were not significantly different from cells treated with vehicle. In cells treated with 1,25(OH)₂D₃ alone or in combination with IFN-γ, PMA-stimulated, 48-kD substrate phosphorylation was increased relative to the other conditions examined. Concomitant exposure of cells to 1,25(OH)₂D₃ and lymphocyte conditioned media did not enhance 48-kD phosphorylation beyond that observed with 1,25(OH)₂D₃ alone (Table 3). Furthermore, no difference in 48-kD phosphorylation was detected when adherent and suspended cells (exposed to the combination of 1,25(OH)₂D₃ and lymphocyte conditioned media) were compared as described in Table 2 (data not shown). The background (PMA-independent substrate phosphorylation) was similar in all conditions examined.

**DISCUSSION**

The U937 cell line has provided a unique model for study of the differentiation and activation of phagocytic cells. By using this cell line it has been possible to demonstrate cell differentiation in response to a wide variety of substances. The active metabolite of vitamin D is 1,25(OH)₂D₃. Receptors for 1,25(OH)₂D₃ have been demonstrated in monocytic phagocytes. We have previously shown that 1,25(OH)₂D₃ enhances differentiation of U937 cells as demonstrated by expression of complement receptors. These effects are most dramatic when 1,25(OH)₂D₃ is combined with lymphokines.

Differentiation of U937 cells also has been associated with the development of the ability of these cells to reduce oxygen, a function important if not critical for microbicidal and tumoricidal activity of mononuclear phagocytes. U937 cell differentiation is associated with competence for nitroblue tetrazolium dye reduction, formation of hydrogen peroxide, and expression of LDL.

The present studies were undertaken to extend our earlier observations regarding the interaction of lymphokines and 1,25(OH)₂D₃. Consistent with these results, we observed maximal competence for LDL among cells exposed to lymphocyte conditioned media plus 1,25(OH)₂D₃. Vitamin D derivatives with less affinity for 1,25(OH)₂D₃ receptors were not equivalent to 1,25(OH)₂D₃. Lymphocyte conditioned media alone acted only as a weak stimulus, and IFN-γ alone or 1,25(OH)₂D₃ alone had no effect in this system.

These results differ somewhat from reports in which U937 cells' ability to reduce O₂ could be demonstrated after their differentiation with either IFN-γ or 1,25(OH)₂D₃ alone. However, in these studies different systems for detecting O₂ reduction were used. Also, clones of U937 cells...
clearly differ in their responses to these factors. In earlier work using the same U937 clone as examined in this report we noted a minimal increase in glucose oxidation and no superoxide release among cells differentiated with 1,25(OH)2D3.4

Several different mechanisms for expression of LDL by phagocytic cells are possible.18 Several different mechanisms for expression of LDL by phagocytic cells are possible. Because light emission was eliminated by SOD, LDL could primarily be attributed to superoxide anion formation. Myeloperoxidase was present in these cells in equivalent concentration under all conditions examined, so differences in myeloperoxidase formation are unlikely to account for differences in LDL observed.20

Formation of superoxide by phagocytic cells involves the transfer of a single electron from NADPH to molecular oxygen.31 This reaction is mediated by an NADPH-dependent oxidase and a unique cytochrome. PMA-mediated O2 reduction has been linked to the transfer of protein kinase activity from the cytosol to a particulate fraction33 and phosphorylation of 43- to 48-kD proteins.22,25 A deficiency in phosphorylation of these substrates has been demonstrated in neutrophils harvested from children with some forms of chronic granulomatous disease of childhood25,26 where the cells are unable to form superoxide. Increased superoxide formation by activated macrophages has also been related to a reduced Km of NADPH oxidase31,32 and enhanced cytochrome function.33

We measured protein kinase C-dependent phosphorylation in U937 cells incubated under conditions known to enhance LDL. The metabolite 1,25(OH)2D3 enhanced maximal protein kinase C-dependent phosphorylation of a 48-kD substrate but did not permit LDL. Lymphocyte conditioned media alone led to LDL but did not enhance 48-kD phosphorylation. Phosphorylation of the 48-kD protein under conditions that allowed maximal LDL, ie, the combination of 1,25(OH)2D3 and lymphocyte conditioned media, was no greater than that of 1,25(OH)2D3 alone. Furthermore, under these conditions LDL but not phosphorylation was greater in adherent cells. These results suggest that although 48-kD phosphorylation may be required for superoxide formation in response to some stimuli the magnitude of phosphorylation cannot be correlated directly with LDL. These results do not preclude the idea that a 1,25(OH)2D3-mediated increase in 48-kD phosphorylation enhances other effects of lymphocyte conditioned media involved in the generation of LDL. Also, it is possible that the conditions of incubation have an effect on translocation of protein kinase C23 or other critical cytosolic components,33 and experiments to examine this hypothesis are in progress.

These results are consistent with our recent work showing activation of human monocyte-derived macrophages by 1,25(OH)2D3.34 In these studies the concentration of 1,25(OH)2D3 required to produce effects was supraphysiological, although serum vitamin D binding proteins reduce the concentration of 1,25(OH)2D3 available to interact with the cell.35 It seems possible that lower concentrations of 1,25(OH)2D3 are permissive for a variety of cell functions.

ACKNOWLEDGMENT

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The metabolite 1 alpha,25-dihydroxyvitamin D3 enhances lymphokine-mediated activation of the oxidative metabolism of the U937 cell line and phosphorylation of a 48-kD respiratory burst-associated protein

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