Phenotypic and Functional Analysis of 1,25-Dihydroxyvitamin D₃ Receptor Mediated Modulation of the Human Myeloma Cell Line RPMI 8226

Jean-François Rossi,² Brian G. M. Durie, Christophe Duperray, Theodore Braich, Samuel L. Marion, J. Wesley Pike, Mark R. Haussler, Charles Janbon, and Regis Bataille


ABSTRACT

Several recent studies have demonstrated the presence of specific receptors for the 1,25-dihydroxyvitamin D₃ (calcitriol) in activated normal lymphocytes. By DNA cellulose chromatography, we show evidence of such specific receptors in the human myeloma cell line RPMI 8226. Nanomolar concentrations of 1,25-dihydroxyvitamin D₃ reduce the proliferation of RPMI 8226 cells significantly and simultaneously induce the appearance of both new properties and phenotype expression, such as butyrate esterase, enhanced expression of CD20 (B1), CD15 (Leu-M1) antigens and λ chains, and decreased expression of the PC1 antigen using microfluorometric analysis. But such an increased expression of membrane λ chains was not associated with an enhanced secretion of λ chains. Furthermore, the bone resorbing activity produced normally by RPMI 8226 cells was reduced significantly after 1,25-dihydroxyvitamin D₃ treatment. The possible mechanisms and significance of these new functional and phenotypic properties are discussed with respect to the B-cell lineage.

INTRODUCTION

Functional cytosolic receptors for 1,25(OH)₂D₃, the most active metabolite of vitamin D₃, were recently discovered in normal leukocytes, including T- and B-activated lymphocytes, monocytes, and macrophages and in their malignant counterparts (1). At nanomolar concentrations, 1,25(OH)₂D₃ but not the other metabolites such as 24,25(OH)₂D₃ and 25(OH)D₃ was found to modulate cell proliferation and differentiation of both normal and malignant cells (2–5). For example, 1,25(OH)₂D₃ is a potent inhibitor of: (a) phytohemagglutinin-induced lymphocyte blast transformation by suppressing the interleukin 2 production, blocking the cell transition from early G₁ to late G₁ cell cycle phase and inhibiting the transferrin receptor expression; (b) secretion of Ig G and M by activated B-cells; (c) HL60 and U937 human cell line growth with differentiation of tumor cells associated to a normal monocyte macrophage phenotype (3, 6–10). Of major interest, the monocye/macrophage differentiation in HL60 occurs in association with the modulation of expression of the c-myc oncogene (11).

RPMI 8226 is a well-established human myeloma cell line (12, 13). Since myeloma cells belongs to the B-cell lineage and since specific receptors for the 1,25(OH)₂D₃ are found on activated normal B-cells and some malignant B-cells, we looked for the presence of such receptors in this human myeloma cell line (1). In this report, we present evidence for the significant expression of such receptors in RPMI 8226. Furthermore, these receptors are found to be functional. Indeed nanomolar concentrations of 1,25(OH)₂D₃ reduce the proliferation of RPMI 8226 cells significantly and induce simultaneously the appearance of new properties and functions, such as the expression of butyrate esterase; enhanced expression of CD20 (B1), membrane light chains, and CD15 (Leu-M1) antigens; decreased expression of PC1 antigen, at the cell surface; and inhibition of secretion of both λ chains and BRA, normally produced by RPMI 8226 cells.

MATERIALS AND METHODS

Cell Culture. RPMI 8226, a continuous myeloma cell line was purchased from the American Type Culture Laboratory (12, 13). The cells were cultured in RPMI 1640 (GIBCO Laboratories, St. Lawrence, MA), with 10% FCS (GIBCO Laboratories), 10 U/ml penicillin, and 100 μg/ml streptomycin (Irvine Scientific, Santa Anna, CA). At least, 30 x 10⁶ cells were harvested while in logarithmic growth phase. The cells were collected, centrifuged, and rinsed three times, then resuspended in 5 ml RPMI 1640 medium supplemented with glutamine and 1% FCS before proceeding for DNA cellulose chromatography.

DNA-Cellulose Chromatography. 1,25(OH)₂[26,27-methyl-³H]cholecalciferol (120 Ci/mmol) was generated as described elsewhere (14). The cells obtained as mentioned above, were incubated for 90 min at 37°C with 2 nM 1,25(OH)₂[³H]D₃. The cells were then centrifugated at 500 x g for 5 min and the pellet resuspended in phosphate buffered saline containing 1% bovine serum albumin. The cells were rinsed twice more in phosphate buffered saline-bovine serum albumin and finally resuspended in 5 ml of 10 mM Tris-Cl, pH 7.5, 2 mM EDTA, 0.5 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 0.15 mM spermidine, and 5 mM diethiothreitol. After 10 min on ice, the cells were lysed by Dounce homogenization and centrifugated for 15 min at 1,100 x g. The crude nuclear pellet thus obtained was resuspended in 2.0 ml KETD-0.3 [1 mM EDTA, 10 mM Tris-Cl, pH 7.4, 5 mM dithiothreitol, and varying concentrations of KCl (i.e., KETD-0.3 = 0.3 mM KCl)] and recentrifugated at 12,000 x g for 10 min. The supernatant resulting was diluted with 4.0 ml KETD-0 in order to reduce ionic strength and this material was then layered onto a 10-ml DNA-cellulose column. The column was eluted at 1 ml/min and 3.0-ml fractions were collected. Receptor bound 1,25(OH)₂[³H]D₃ was then eluted from the column by means of a 100-ml KETD 0.1–0.5 ml linear gradient. All these operations were carried out at 4°C. A 0.5-ml aliquot of each fraction was counted in 5.0 ml of ACS (Amersham Corp., Arlington Heights, IL) on a Beckman LS-230 scintillation counter. Gradient molarity was determined with a conductivity meter (Radiometer, Copenhagen, Denmark).

Cell Proliferation in Liquid Culture. 1 x 10⁸/ml RPMI 8226 cells were cultured as mentioned above, in the presence of various concentrations of 1,25(OH)₂D₃ (10⁻⁷–10⁻¹¹ M) (supplied by Dr. Milan Uskokovic of Hoffman-LaRoche, Nutley, NJ). Control cultures contained the ethanol vehicle at 0.1% (vol/vol). Seven flasks (25-cm²) flasks; Falcon, Becton Dickinson, Oxnard, CA) were prepared for each concentration of 1,25(OH)₂D₃. Each flask was sampled in sterile fashion and counted after shaking to collect any adherent cells at days 2 and 4. Cell viability determined by trypan blue exclusion was ≥95%. The titiated thymidine

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2 To whom requests for reprints should be addressed, at Unité Inserm U291, 99, rue Puech Villa-Zolad, 34100 Montpellier, Cedex, France.

3 The abbreviations used are: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; Ig, immunoglobulin; BRA, bone resorbing activity; FCS, fetal calf serum; CD, clusters of differentiation.
cell proliferation in agar semisolid medium. The culture system has been previously described in detail (16). Briefly, the cells to be tested were suspended in 0.3% agar in enriched CMRL 1066 medium, supplemented with 15% horse serum in order to yield a final concentration in the range of 2.5 × 10^4 cells/mL. Freshly prepared 2-mercaptoethanol was added at a concentration of 5 × 10^-4 M immediately before triplicate plating of the cells. One milliliter of the mixture was pipetted into a 35-mm plastic Petri dish containing conditioned medium in a 1-ml agar feeder layer. Because the plating efficiency in a myeloma was 0.01–0.2%, a plating concentration of 5 × 10^5 cells was normally used. Cultures were incubated at 37°C in an incubator. Colonies (collections of more than 30 cells) appeared within 10 to 21 days, and plates were counted for 1,25(OH)₂D₃ effects after 14 to 21 days, using an inverted-phase microscope.

Cell Maturation and Morphology. The RPMI 8226 cells were cultured with and without 1,25(OH)₂D₃ (concentrations of 10⁻⁷ and 10⁻¹⁰ M), medium containing 10% FCS, as detailed above. Cyto centrifuge preparations were made and histochemical stains (Wright-Giemsa, nonspecific esterase, β-glucuronidase, acid phosphatase) were performed, as previously described (17–19). Reactivity of the 8226-treated cells [10⁻⁷ M, 1,25(OH)₂D₃] with murine monoclonal antibodies [CD15 (Leu-M1), CD20 (B1), PC1 (Becton Dickinson, Sunnyvale, CA), CD17 (G035), and CD23 (HD50, TU1) were obtained from the panel of the Third International Workshop on the Differentiation Leukocytes Antigens] was assayed by an indirect immunofluorescence technique using both fluorescence-activated cell sorting and avidin-biotin peroxidase techniques, as previously described (20, 21).

Bone Resorbing Activity (BRA) Bioassay. 5 × 10⁶ RPMI 8226 cells were cultured in 10 ml RPMI 1640 supplemented with glutamine and 1% FCS for 2 days with and without different concentrations of 1,25(OH)₂D₃ (10⁻⁷–10⁻¹⁵ M). The cells from each sample were washed twice in medium and recultured in RPMI 1640 without any FCS for one more day. Supernatants were then collected and frozen at −70°C until use. BRA bioassay was made as previously described (23, 24). Briefly, pregnant rats were injected i.p. with ⁴⁵Ca on the 18th day of gestation. On the following day, the rats were killed, the fetuses removed and the long bones dissected free of soft tissues. The bones were then preincubated for 24 hr in control medium in order to enable exchange of loosely bound ⁴⁵Ca. The bones were then cultured either in control medium or conditioned medium (supernatants previously prepared). After 2 days, ⁴⁵Ca released from bone into medium was measured and a ratio of ⁴⁵Ca released into conditioned versus control medium generated. For each sample, eight to 12 equal pairs of bones were available. For statistical analysis, we used the Wilcoxon rank test for matched pairs (two-tailed test).

RESULTS

Detection and Quantitation of 1,25(OH)₂D₃ Receptors on the RPMI 8226 Cell Line. To extend the notion of presence of 1,25(OH)₂D₃ receptors further in normal activated B-cells and malignant B-lymphoblastoid cells, we attempted first to determine whether 1,25(OH)₂D₃ receptors were present on RPMI 8226 human plasma cell line. As illustrated in Fig. 1, the majority of bound 1,25(OH)₂D₃ eluted in a single peak at 0.23 M KCl, characteristic of 1,25(OH)₂D₃ receptors (3, 25). Integrated analysis of this DNA-binding peak yielded a 1,25(OH)₂D₃ receptor value of 2600 copies of receptor/cell.

Marked Studies. After 1,25(OH)₂D₃ treatment, RPMI 8226 cells show some changes in morphology and in phenotype (Table 1). This includes the expression of nonspecific esterase in 25% of the treated cells (versus 0% of the nontreated cells), a slight increase in the percentage of CD23 (A-B cell marker) and CD15 (CD15)-positive cells, especially observed on huge cells, and a slight decrease in the percentage of CD17 positive cells. The major changes observed were the appearance of 23% CD20 (B1)-positive cells, the increase in membrane light chain-positive cells and the decrease in PC1-positive cells. There was no significant change in acid phosphatase or β-glucuronidase staining, even at the highest 1,25(OH)₂D₃ concentrations at Day 2 or 4. None of the T-cell antigens, including interleukin 2 receptor, nor Calla (CDIO), nor CD5 (Leu-1, Becton Dickinson) were expressed after 1,25(OH)₂D₃ exposure (data not shown).

Growth Response and Morphology Studies. In order to test the functional capacity of the 1,25(OH)₂D₃ receptors present on the RPMI 8226 cell line, we studied the effect of the 1,25(OH)₂D₃ on cell proliferation, phenotype, and functional activity such as bone resoring activity. Fig. 2 shows the dose response effect of 1,25(OH)₂D₃ upon the growth of RPMI 8226 cells in liquid culture. As mentioned above, significant inhibition of growth was seen at concentrations as low as 10⁻⁹ M vitamin D₃ at Day 2 (p < 0.01) and inhibitory effects remained significant at 10⁻⁸ M up to 4 days. This difference may be due to the stability of the drug in the medium. There was also a striking decrease in the number of colonies when tumor cells were grown in semisolid agar medium, with different concentrations of 1,25(OH)₂D₃ (Fig. 3). Significant growth inhibition was also associated with a 20–30% reduction in the tritiated thymidine labeling index with concomitant reductions in grains/cell (data not shown).

Bone Resorbing Assay. RPMI 8226 cells are known to secrete BRA (26, 30). After 1,25(OH)₂D₃ treatment, RPMI 8226 cells were unable to secrete such BRA in comparison with nonactivated 8226 cells cultured in the same conditions and showing a BRA (Table 2).

DISCUSSION

In this study, we have demonstrated that receptors for 1,25(OH)₂D₃ are present on RPMI 8226, a well-studied EBV-negative human myeloma cell line. To our knowledge, this is the first demonstration of 1,25(OH)₂D₃ receptors in the myeloma plasma cell line. Combining this information with previously published data indicating that receptors are present on
normal activated B-cells as well as on some malignant B-cells, it is tempting to conclude that receptors for 1,25(OH)_2D_3 are present throughout the various stages of the normal maturation of the human B-activated lymphocytes, including the terminal stage, i.e., the Ig-secreting cell or plasma cell. Of additional importance, these receptors were found to be functional with doses of dexamethasone in multiple myeloma (28). Further more, other steroid receptors, such as estrogen receptors have been recently demonstrated on myeloma cell lines in RPMI 8226 particularly (29). The current data is too limited to speculate about the clinical utility of 1,25(OH)_2D_3 in patients with myeloma. However, this drug has already been tested in both mice and humans, for modulation of the growth and the differentiation of myeloid leukemic cells (1–4). It also suggests that 1,25(OH)_2D_3 can modulate gene expression (including oncogenes such as c-myc in HL60 cell line, which is not amplified in RPMI 8226)'s and proteins synthesis.

Table 2  Inhibition of BRA secretion by 1,25(OH)_2D_3-treated RPMI 8226 cells

<table>
<thead>
<tr>
<th>RPMI 8226 cell line</th>
<th>BRA*</th>
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<tbody>
<tr>
<td>Nontreated cells</td>
<td>1.26 ± 0.11 (NS)</td>
</tr>
<tr>
<td>10^-7 M 1,25(OH)_2D_3-treated cells</td>
<td>0.94 ± 0.15 (NS)</td>
</tr>
<tr>
<td>10^-8 M 1,25(OH)_2D_3-treated cells</td>
<td>0.81 ± 0.14 (NS)</td>
</tr>
<tr>
<td>10^-9 M 1,25(OH)_2D_3-treated cells</td>
<td>0.92 ± 0.16 (NS)</td>
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<tr>
<td>10^-10 M 1,25(OH)_2D_3-treated cells</td>
<td>1.05 ± 0.19 (NS)</td>
</tr>
<tr>
<td>10^-11 M 1,25(OH)_2D_3-treated cells</td>
<td>1.11 ± 0.03 (NS)</td>
</tr>
</tbody>
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* See “Materials and Methods” and Ref. 23.

REFERENCES
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