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

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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)2D3,1 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) phytodemetabolitin-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 monocyte/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 × 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[26,27-3H]cholecalciferol (120 Ci/mmole) 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 centrifuged at 100 × 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-HCl, pH 7.5, 2 mM EDTA, 0.5 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 0.15 mM spermidine, and 5 mM dithiothreitol. After 10 min on ice, the cells were lysed by Dounce homogenization and centrifugated for 15 min at 1100 × g. The supernatant obtained was then centrifugated at 12,000 x g for 10 min. The supernatant resulting was diluted with 4.0 ml KETD-0 in order to 1% KETD-0 and recentrifugated at 12,000 × 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/mm and 3.0-M KCl gradient. All these operations were carried out at 4°C. 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 centrifuged at 100 × 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-HCl, pH 7.5, 2 mM dithiothreitol, and varying concentrations of KCl (i.e., KETD-0.3 = 0.3 M KCl) and centrifugated at 12,000 × 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-M 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-mi 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 × 105/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...
labelling index was performed, as previously described (15).

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 × 10^5 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)_2D_3 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)_2D_3 (concentrations of 10^-9 and 10^-11 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)_2D_3] 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 × 10^5 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)_2D_3 (10^-9–10^-11 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 (22) and modified by ourselves (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 precultured for 24 h 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)_2D_3 Receptors on the RPMI 8226 Cell Line. To extend the notion of presence of 1,25(OH)_2D_3 receptors further in normal activated B-cells and malignant B-lymphoblastoid cells, we attempted first to determine whether 1,25(OH)_2D_3 receptors were present on the RPMI 8226 human plasma cell line. As illustrated in Fig. 1, the majority of bound 1,25(OH)_2D_3 eluted in a single peak at 0.23 M KCl, characteristic of 1,25(OH)_2D_3 receptors (3, 25). Integrated analysis of this DNA-binding peak yielded a 1,25(OH)_2D_3 receptor value of 2600 copies of receptor/cell.

Marker Studies. After 1,25(OH)_2D_3 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 controls), a slight increase in the percentage of CD23 (B cell marker) and Leu-M1 (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 A 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)_2D_3 concentrations at Day 2 or 4. None of the T-cell antigens, including interleukin 2 receptor, nor Calla (CD10), nor CD5 (Leu-1, Becton Dickinson) were expressed after 1,25(OH)_2D_3 exposure (data not shown).

Growth Response and Morphology Studies. In order to test the functional capacity of the 1,25(OH)_2D_3 receptors present on the RPMI 8226 cell line, we studied the effect of the 1,25(OH)_2D_3 on cell proliferation, phenotype, and functional activity such as bone resorbing activity. Fig. 2 shows the dose response effect of 1,25(OH)_2D_3 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 D, at Day 2 (p < 0.01) and inhibitory effects remained significant at 10^-4 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)_2D_3 (Fig. 3). Significant growth inhibition was also associated with a 20–30% reduction in the tritiated thymidine labeling index with concomittant reductions in grains/cell (data not shown).

Bone Resorption Assay. RPMI 8226 cells are known to secrete BRA (26, 30). After 1,25(OH)_2D_3 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)_2D_3 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)_2D_3 receptors in the myeloma plasma cell line. Combining this information with previously published data indicating that receptors are present on
Activation of the 1,25(OH)₂D₃ receptors on the RPMI 8226 cell line modulates some functions such as the secretion of BRA, a property of this cell line (23, 26). This inhibition has to be evaluated with respect to other actions of the vitamin D₃, such as the reduction in the interleukin 2 and Ig production by activated B-cells (6, 8, 9). We also demonstrated a reduction in light chain synthesis by these activated cells (data not shown).

All of these decreased activities are probably the result of an effect at the DNA level with gene modulation, as previously demonstrated for the c-myc oncogene (11). Two aspects of our present data deserve special comment. Firstly, steroid receptors (dexamethasone) have previously been demonstrated on the RPMI 8226 cell line and the growth of RPMI 8226 cells is known to be inhibited by low concentrations of dexamethasone, with maximum drug binding occurring during the S-phase of the cell cycle (27). It is not known if this is associated with the induction of a new surface phenotype on the myeloma cells. The sensitivity of myeloma cells to dexamethasone in vitro is supported by in vivo data showing the antitumor effect of high doses of dexamethasone in multiple myeloma (28). Furthermore, 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)₂D₃ 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)₂D₃ can modulate gene expression (including oncogenes such as c-myc in HL60 cell line, which is not amplified in RPMI 8226) and proteins synthesis.

REFERENCES


Table 2 Inhibition of BRA secretion by 1,25(OH)₂D₃-treated RPMI 8226 cells

<table>
<thead>
<tr>
<th>Concentration of 1,25(OH)₂D₃ (Molar)</th>
<th>Number of Cells x 10⁶/ml</th>
<th>BRA*</th>
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<tbody>
<tr>
<td>Control</td>
<td>50</td>
<td>100</td>
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<tr>
<td>10⁻¹⁰ M 1,25(OH)₂D₃-treated cells</td>
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<td>90</td>
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<td>10⁻⁸ M 1,25(OH)₂D₃-treated cells</td>
<td>40 ± 2 (NS)</td>
<td>80</td>
</tr>
<tr>
<td>10⁻⁶ M 1,25(OH)₂D₃-treated cells</td>
<td>35 ± 1 (NS)</td>
<td>70</td>
</tr>
</tbody>
</table>

*See "Materials and Methods" and Ref. 23.


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