Specific Uptake of 1,25-Dihydroxycholecalciferol by Human Chronic Myeloid Leukemia Cells

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ABSTRACT

We have examined mononuclear cell preparations from patients with chronic myeloid leukemia [CML] for binding of and response to 1,25-dihydroxycholecalciferol [1,25-(OH)2D3]. Whole cells specifically took up [3H]-1,25-(OH)2D3 with high affinity (Kd 3.6 x 10^-11 M) and low capacity. Subcellular fractionation of labeled cells showed that binding was restricted to cytosols and nuclei. Sucrose gradient centrifugation of cells preincubated with [3H]-1,25-(OH)2D3 revealed a single 3.6S peak which was totally displaced with 100-fold excess nonradioactive hormone. However, we were unable to demonstrate specific binding of 1,25-(OH)2D3 by postlabeling standard cytosol preparations. In addition, cytosols prepared from a mixture of CML cells and 1,25-(OH)2D3 receptor-positive T47D (human breast cancer) cells had less than 10% of the binding measured in T47D cytosol alone. However, the levels of binding in T47D cytosols were not reduced if the receptors were occupied with [3H]-1,25-(OH)2D3 prior to the addition of the CML cytosols. Thus, CML cells appear to contain both the receptor for 1,25-(OH)2D3 and an unknown substance which prevents its detection following the preparation of cytosol. Cells from patients with CML in the chronic phase specifically bound more 1,25-(OH)2D3 than did cells from patients with acute myeloid leukemia [AML] (2.6 ± 1.5 fmol/10^7 cells) than did those in acute myeloid transformation [7.2 ± 1.5] or than did cells from patients with acute myeloid leukemia [2.6 ± 0.8]. Only cells from the first group of patients responded to the addition of 1,25-(OH)2D3 by differentiating along the monocyte-macrophage pathway. We conclude that the differentiation-induction effect of 1,25-(OH)2D3 is likely to depend on adequate levels of receptor and that intact cells rather than cytosol preparations should be studied before cells of a particular tissue are designated as receptor negative.

INTRODUCTION

Recently, studies on the distribution of the specific binding protein for 1,25-(OH)2D3 have suggested several new sites of action for this calcium-regulating hormone. Ten years ago, intestine, bone, and kidney were the only target tissues known, but now the list includes other calcium-transporting organs (breast, placenta), endocrine glands producing hormones thought to regulate the renal 25-OH-D3 hydroxylases (parathyroids, pituitary, ovary, etc.), and other sites where the function of 1,25-(OH)2D3 is more obscure (skin, brain) (13).

1,25-(OH)2D3 receptors are also present in a number of neoplastic tissues and in cell lines derived from them (4–6, 9). The presence of the receptor in transformed cells has generally been assumed to represent a retained property of the parent tissue. Biological effects of 1,25-(OH)2D3 in cancer cells have been harder to demonstrate, although effects on proliferation in breast (8), melanoma (9), and bone (11) cells in culture have now been seen.

In contrast, prior to the demonstration of a receptor, Abe et al. (1) found that 1,25-(OH)2D3 induced monocytic differentiation in a murine leukemia cell line (M1). They also reported that 1,25-(OH)2D3 caused granulocytic differentiation of cells from a human promyelocytic leukemia cell line (HL60) and showed that these cells possessed the cytosolic receptor for 1,25-(OH)2D3 (19). The biological effects produced by different cholecalciferol metabolites in these cells correlated well with their affinity for the 1,25-(OH)2D3 receptor. The differentiation of these cells was accompanied by a slowing of proliferation, and the potential significance of this has been emphasized recently by the same group’s demonstration of prolonged survival times in mice given injections of M1 cells, when treated with 1,25-(OH)2D3 or 1α-hydroxycholecalciferol (10).

In our laboratory, we have found that 1,25-(OH)2D3 causes monocytic differentiation of HL60 cells, as judged by morphology, cytochemistry, and the presence of specific surface antigens. We have also seen similar effects in normal bone marrow (12). In addition, Bar-Shavit et al. (2) have recently demonstrated 1,25-(OH)2D3-induced monocytic differentiation of HL60. We here report the characterization of specific 1,25-(OH)2D3 uptake and response of mononuclear cells from patients with myeloid leukemias.

MATERIALS AND METHODS

[3H]-1,25-(OH)2D3 (specific radioactivity, 163 Ci/mmol) was purchased from Amersham International (Amersham, Buckinghamshire, United Kingdom). Nonradioactive cholecalciferol metabolites were a gift from Roche Products (Welwyn Garden City, Hertfordshire, United Kingdom). Reagents were of analytical grade and were obtained from commercial suppliers. Bone marrow or buffy coat cells were obtained from patients with Philadelphia chromosome-positive CML when they were either in the chronic stage of their disease or in acute myeloid transformation (17). Cells were also obtained from patients with AML. The mononuclear fraction was prepared by layering the cell suspension onto Lymphoprep (Nyegaard, Ltd., Oslo, Norway), centrifuging at 400 x g for 25 min, and collecting the cells at the interface.

Cytosol Binding Studies. Cells were initially examined for specific
cytosolic binding of 1,25-(OH)2D3. Cytosols were prepared by sonicating cells (2 to 5 x 10^7/ml) on ice in a Tris buffer (KTEDMT) known to optimize 1,25-(OH)2D3 receptor measurement in other systems (20). The homogenates were centrifuged at 88,000 x g for 30 min at 4°C in the 70Ti rotor of a Beckman L8 70 ultracentrifuge. Binding of [3H]-1,25-(OH)2D3 was assayed in these cytosols as described previously (8).

**Whole-Cell Uptake.** Specific uptake of [3H]-1,25-(OH)2D3 by intact cell preparations was determined largely as described by Sher et al. (18), except that a shorter incubation time was found to be sufficient to maximize binding. Cells (5 x 10^6) were incubated in triplicate for 3 hr at 37° in 0.5 ml RPMI 1640 plus 0.05% BSA with 0.1 pmol [3H]-1,25-(OH)2D3 in the presence or absence of 10 pmol 1,25-(OH)2D3 or 25-OHD3. At the end of the incubation, cells were spun down (800 x g, 5 min) and resuspended in 0.5 ml phosphate-buffered saline (8 g NaCl, 0.2 g KCl, and 0.2 g NaH2PO4 in 100 ml distilled H2O) containing 0.5% (w/v) BSA at 20 min at room temperature. The suspensions were pelleted, rinsed once more in phosphate-buffered saline alone, and then dissolved in 0.5 ml 1 M NaOH. The pH was corrected to 7.4 with glacial acetic acid, prior to counting in 10 ml scintillant ES299 (Packard Instruments, Caversham, Berkshire, United Kingdom) using a Packard Tri-Carb liquid scintillation counter with an efficiency for tritium of 30%. Further competition studies were performed on some preparations by incubating cells, as described above, with 0.06 pmol [3H]-1,25-(OH)2D3 and increasing amounts of cholecalciferol, its metabolites, or other steroids (10^-6 to 10^-10 M).

The affinity between CML cells and [3H]-1,25-(OH)2D3 was determined by incubating cells (5 x 10^6) with increasing amounts (0.01 to 0.15 pmol) of [3H]-1,25-(OH)2D3 for 3 hr at 37°; parallel incubations in the presence of 100-fold excess nonradioactive 1,25-(OH)2D3 were used to determine nonspecific uptake. Cells were processed prior to liquid scintillation counting as described above. Data were analyzed by the method of Scatchard (16).

In addition, cytosols were prepared as before from cells prelabeled with 0.1 pmol [3H]-1,25-(OH)2D3 ± 10 (S.E.) pmol, 1,25-(OH)2D3, or 25-OHD3 at 37° for 3 hr. The cytosols were applied to 5 to 20% sucrose gradients (w/v) in KTEDMT and spun at 288,000 x g for 15 hr at 4°C in the SW55 rotor of a Beckman L870 ultracentrifuge. Gradients were divided into 150-μl fractions from the bottom by downward displacement with mineral oil.

**Subcellular Localization.** Cells which had been incubated with tritiated hormone and rinsed as described were homogenized by resuspending in hypotonic buffer (TEDT), followed by 10 passages through a 25-gauge needle. This procedure lysed more than 95% of the cells. KCI in TEDT was added to give a final concentration of 0.15 M KCl. Homogenates were spun at 800 x g for 5 min. The supernatants were recentrifuged at 88,000 x g for 30 min to yield a clear cytosol and a pellet which was presumed to consist largely of mitochondria and microsomes. The 800 x g pellet was rinsed, centrifuged, and suspended in 1 ml sucrose in TEDT. This was layered onto a sucrose pad (1.7 M sucrose in TEDT) and centrifuged at 30,000 x g for 20 min. The sucrose buffers were aspirated to leave a pellet of semipurified nuclei, which were resuspended in KTEDMT and incubated on ice for 20 min. They were recentrifuged at 800 x g, and the radioactivities in the supernatant and pellets were counted separately.

**Combination Experiments.** In an attempt to explain the apparent lack of specific [3H]-1,25-(OH)2D3 binding in CML cytosols, specific binding of the hormone to cytosols prepared from a receptor-positive human breast cancer cell line (T47D (8)) was measured alone or in combination with CML cytosol. T47D cells were grown as described previously (8), suspended in KTEDMT (2 x 10^7 cells/ml) and mixed with either CML cells (5 x 10^6 cells/ml) or an equal volume of BSA (1 mg/ml). The cell suspensions were sonicated, and cytosols were prepared and assayed for specific [3H]-1,25-(OH)2D3 binding as before.

**Cell Differentiation.** Cells from patients with CML or AML were incubated with 10^-6 to 10^-8 M 1,25-(OH)2D3 or 25-OHD3 as described previously for normal bone marrow and HL60 cells; they were characterized.

**RESULTS**

Despite clear evidence of biological activity of 1,25-(OH)2D3 in CML cells, only low to undetectable levels of specific binding (<200 cpm/10^6 cells) could be measured in cytosol preparations, using buffers and assay conditions found optimal for the receptor measured in other cytosols. The level of binding was not enhanced by increasing the concentration of protease inhibitor (Trasylol) in the buffer. As a consequence, the specific uptake of [3H]-1,25-(OH)2D3 by intact cells was examined.

CML cells incubated at 37° accumulated [3H]-1,25-(OH)2D3 in a time-dependent manner, reaching a maximum by 2 hr, which was maintained for at least 6 hr. This uptake was partially displaceable by 100-fold excess 1,25-(OH)2D3 but not by 25-OHD3. Competition studies using multiple concentrations of cholecalciferol metabolites showed that both 25-OHD3 and 24,25-dihydroxycholecalciferol (D3), or cholecalciferol (D3). Similar results were seen with cells from 3 other CML patients in chronic phase.

**Chart 1.** Competition for [3H]-1,25-(OH)2D3 uptake into whole CML cells. CML cells (5 x 10^6) were incubated in duplicate for 3 hr at 37° with 0.06 pmol [3H]-1,25-(OH)2D3 alone or in the presence of various amounts (10^-10 to 10^-15 M) of cholecalciferol or its metabolites. Cells were rinsed twice, and the amount of radioactivity contained in them was assayed by liquid scintillation counting. Results are expressed as percentage maximum specific binding, i.e., the difference between binding in the absence or presence of a 100-fold excess of nonradioactive 1,25-(OH)2D3. Competing steroids were 1,25-(OH)2D3 (A), 25-OHD3 (B), 24,25-dihydroxycholecalciferol (D3), or cholecalciferol (D3). Similar results were seen with cells from 3 other CML patients in chronic phase.

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cytosols, mitochondria-microsomes, 0.3 mM KCl nuclear extracts, and residual nuclei. Specific binding was restricted to cytosols (29%), nuclear extracts (29%), and nuclear residues (41%).

Similarly, labeled cells were homogenized by sonication in hypertonc buffer, cytosols were prepared, and the radioactivity in them was analyzed by sucrose density gradient ultracentrifugation. A single peak was seen, migrating one fraction later than the 3.7S marker, which was totally abolished when cells were preincubated with excess 1,25-(OH)₂D₃ but only slightly reduced in the presence of excess 25-OHD₃ (Chart 3). No peak was visible when cytosols were incubated directly with tritiated hormone.

The specific uptake of [³H]-1,25-(OH)₂D₃ by CML cells thus appeared to be due to a binding moiety with the characteristics of the 1,25-(OH)₂D₃ receptor found in other tissues. In an attempt to discover why the receptor was not measurable in standard cytosol preparations, such cytosols were mixed with those from the receptor-positive T47D cells and binding assessed. Specific binding was not detected in CML cytosols alone. Cytosols obtained from a mixture of T47D and CML had only 5 to 10% of the specific binding seen in cytosols prepared from T47D cells alone (Chart 4). This loss of binding was seen whether homogenates were prepared by hypotonic lysis or sonication and also, although to a slightly reduced extent, when binding was assayed at 4°, rather than at 25°. T47D cells were also prelabeled with [³H]-1,25-(OH)₂D₃ mixed with CML cells and homogenized; then, cytosols were prepared and incubated under the same time and temperature conditions. The levels of specific binding in T47D cytosols under these conditions were not reduced by the pres-

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**Chart 2.** Saturation analysis of [³H]-1,25-(OH)₂D₃ uptake by CML cells. CML cells (5 x 10⁶) were incubated in duplicate with increasing amounts (0.01 to 0.15 pmol) of [³H]-1,25-(OH)₂D₃ alone (A, total binding) or in the presence of 100-fold excess nonradioactive 1,25-(OH)₂D₃ (A, nonspecific binding) for 3 hr at 37°. The difference in uptake between the 2 (■, specific binding) was used to generate the Scatchard plot shown on the right. Kᵣ = 3.6 x 10⁻¹¹ M, Nmax = 29.3 fmol/10⁷ cells.

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**Chart 3.** Sucrose density gradient analysis of [³H]-1,25-(OH)₂D₃ binding in CML cells. CML cells were incubated with [³H]-1,25-(OH)₂D₃ (0.1 pmol) alone (□) or in the presence of 10 pmol 1,25-(OH)₂D₃ (○) or 25-OHD₃ (△). Cells were rinsed twice, and cytosols were prepared by sonication in KTEDMT, followed by ultracentrifugation. In addition, cells were homogenized, and then the cytosols were incubated with [³H]-1,25-(OH)₂D₃ (■). All cytosols were applied to 5 to 20% sucrose gradients and centrifuged at 288,000 x g for 14 hr at 4°. They were divided into 150-μl fractions from the bottom. Arrow, position of 3H-ovalbumin (3.7S) run in a parallel gradient. T, top fraction; B, bottom fraction.

**Chart 4.** Effect of CML cytosol on specific [³H]-1,25-(OH)₂D₃ binding in T47D cytosol. Cytosols were prepared from T47D cells alone (□) or in combination with CML cells (△). Binding of [³H]-1,25-(OH)₂D₃ to unoccupied receptors was assessed in the absence or presence of 100-fold excess 1,25-(OH)₂D₃ at either 25° (40 min) or 4° (3 hr), with separation of unbound steroid by charcoal. For occupied receptors, T47D cells were prelabeled with [³H]-1,25-(OH)₂D₃ ± 100-fold excess 1,25-(OH)₂D₃, and cytosols prepared with or without the addition of CML cells were incubated for 40 min at 25° and then charcoal treated. Columns, mean of triplicate estimations; bars, S.E.
ence of CML cytosols (Chart 4), thus suggesting that occupation of the 1,25-(OH)2D3 receptor prevents its inactivation by CML cytosols. The studies reported above were performed using mononuclear cells from patients with CML in the chronic phase of their disease. Using a single saturating dose assay, specific uptake of 1,25-(OH)2D3 by cells of this type was 18.0 ± 3.2 fmol/107 cells (n = 6). Cells taken from patients with the same disease, but in acute transformation, had only one-half this level of specific uptake, and those from patients with AML accumulated still less 1,25-(OH)2D3 (Table 1). CML cells in chronic phase reproducibly differentiated along the monocyte-macrophage pathway in response to 1,25-(OH)2D3 as judged by a fluorode-sensitive positive reaction with α-naphthyl acetate esterase (Table 1). Although, as previously observed in AML (14), cells from patients with CML blast crisis or AML showed some spontaneous monocytic differentiation upon culture in vitro, this differentiation was not enhanced by 1,25-(OH)2D3.

DISCUSSION

The uptake of 1,25-(OH)2D3 by mononuclear cells from CML patients is of high affinity and specific to this active metabolite of cholecalciferol. Furthermore, homogenization of cells prelabeled with hormone showed that the specifically bound radioactivity was restricted to cytosols and nuclei. Sucrose density centrifugation of high-salt cytosols allowed the detection of a 3.5- to 3.7S peak which was totally abolished when cells were preincubated with 100-fold excess 1,25-(OH)2D3, but only slightly reduced when 25-OHD3 was the competitor. These data, particularly when considered in conjunction with our evidence for biological responses to 1,25-(OH)2D3, clearly suggest that CML cells contain the specific 1,25-(OH)2D3 receptor with the same characteristics as those seen in classic target tissues (7, 13, 21).

Our failure to detect this protein in standard cytosol preparations suggested that homogenization of CML cells led to the inactivation of 1,25-(OH)2D3 receptors, either by proteolytic degradation, by chemical modification, or by blocking of the binding site by an alternative endogenous ligand. We therefore investigated the effects of addition of CML cytosol to the 1,25-(OH)2D3 receptor content of T47D cell cytosols. This led to a reduction of approximately 90% in the levels of specific binding measured in T47D cytosols. The loss of binding occurred at 4°C as well as at 25°C, and increasing the concentration of Trayloll in the buffer did not permit the detection of receptor in CML cytosols. It therefore seemed unlikely that enzymatic degradation was responsible for the receptor loss. When receptors in T47D cells were occupied with [3H]-1,25-(OH)2D3 prior to the addition of CML cytosol, a reduction in the binding subsequently assayed did not occur. Thus, the inactivation process is likely to involve parts of the receptor molecule at or near the 1,25-(OH)2D3 binding site, which are protected in the presence of the ligand. The exact nature of this process awaits further investigation; a number of agents are known which inactivate the unoccupied 1,25-(OH)2D3 receptor, while not affecting the hormone-bound form, e.g., the sulfhydryl alkylating agent N-ethylmaleimide (21).

If our interpretation of these results is correct and CML cells contain 1,25-(OH)2D3 receptors which are not routinely measurable in cytosols, it is possible that other cells may possess receptors which have not been detected for similar reasons. Previous quantitation of receptors in established target tissues may be inaccurate, since the levels measured would represent a balance between the intrinsic content and the inactivating capacity of the tissue. However, addition of cytosol prepared from breast tumors does not appear to diminish the levels of receptor seen in T47D cytosols (5).

Cells from patients with either AML or CML in acute transformation take up less 1,25-(OH)2D3 than CML cells in chronic phase. In addition, these more primitive cells do not, in our experience, differentiate in response to 1,25-(OH)2D3. This suggests that possession of adequate levels of receptor is necessary for the occurrence of the biological effect. It also suggests that the amount of receptor present in myeloid malignant cells varies with their state of maturity and that more primitive, less differentiated cells have not yet developed their capacity to respond to 1,25-(OH)2D3.

Preliminary results indicate that a similar receptor exists in marrow cells taken from normal donors. This is, in any case, likely since the distribution of myeloid cell types in chronic-phase CML marrow is similar to that found in normal marrow (17). The determination of which particular normal and transformed human blood cells are targets for 1,25-(OH)2D3 is now proceeding. When normal or leukemic progenitor cells from human bone marrow differentiate into more mature forms, their ability to proliferate usually decreases. Consequently, some approaches to leukemia therapy are now directed towards differentiating the tumor cells rather than simply killing them (15). 1,25-(OH)2D3 inhibits proliferation of HL60 cells in vitro (12, 19). The growth of M1 mouse myeloid leukemia cells is also inhibited by 1,25-(OH)2D3 in vitro (1), and the same phenomenon occurs in vivo (10). Thus, the experimental use of 1,25-(OH)2D3 in therapy of some forms of leukemia and preleukemia must now be considered.

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