Leukemia Research 34 (2010) 649–657

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Different susceptibilities to 1,25-dihydroxyvitamin D_3-induced differentiation of AML cells carrying various mutations

Elżbieta Gocek[a], Marek Kiełbiński[b], Hanna Baurska[a], Olga Haus[b,c], Andrzej Kutner[d], Ewa Marcinkowska[a,*]

[a] Department of Biotechnology, University of Wrocław, Tamka 2, 50-137 Wrocław, Poland
[b] Department of Hematology, Blood Neoplasms and Bone Marrow Transplantation, Wrocław Medical University, Wrocław, Poland
[c] Department of Clinical Genetics, Collegium Medicum UMK, Bydgoszcz, Poland
[d] Pharmaceutical Research Institute, Warszawa, Poland

ARTICLE INFO

Article history:
Received 1 June 2009
Received in revised form
12 September 2009
Accepted 4 October 2009
Available online 31 October 2009

Keywords:
Acute myeloid leukemia
Cell lines
Differentiation
1,25-Dihydroxyvitamin D_3
Vitamin D analogs
Vitamin D receptor
Flow cytometry
Western blotting

ABSTRACT

This study was designed to compare the differentiation-inducing potential of 1,25-dihydroxyvitamin D_3 (1,25D) with some analogs (VDAs) in a panel of acute myeloid leukemia (AML) cell lines and in blast cells isolated from patients with AML. Of the cell lines studied, HL60 proved to be the most sensitive to each of the differentiation-inducing agents when compared to THP-1, NB-4 and U-937 cell lines. Three of the VDAs tested (PRI-1906, PRI-2191 and PRI-2201) were similarly effective as 1,25D in all the cell lines tested. However, blast cells from AML showed a varying sensitivity towards 1,25D. For example, blast cells isolated from patients in which the whole or part of chromosome 7 was deleted were extremely sensitive to 1,25D and its analogs. In contrast, 1,25D failed to increase the expression of differentiation markers in blast cells isolated from patients carrying activating mutations in *Flt3* gene. Since, the expression of vitamin D receptor (VDR) in cells with *Flt3* mutations was increased to the same extent as in other AML cells this suggests that failure of these cells to differentiate lies downstream of the receptor. That blast cells with different cytogenetic abnormalities have dissimilar responses to 1,25D and its analogs, may have implications in the use of 1,25D as a 'differentiation therapy' for myeloid leukemias. The analog PRI-2191 (tacalcitol) was found to be the most potent in inducing patient's cells differentiation.

1. Introduction

Acute myeloid leukemias (AMLs) are heterogeneous group of blood malignancies characterized by a block at various stages of hematopoietic differentiation, leading to the accumulation of immature myeloid cells in bone marrow and peripheral blood. Despite significant improvements in chemotherapeutic regimens, poor responsiveness and relapse are still problems in a significant number of myeloid leukemic patients. With chemotherapy alone clinical outcome for many myeloid leukemic patients can still be poor. Therefore, the management of AML presents significant challenges, and there is still a need for new therapies with greater efficacy and better tolerability than existing treatments [1]. An alternate way to treat myeloid leukemia was suggested over 30 years ago, which is called now 'differentiation therapy' [2–4]. In *in vitro* studies, it was noticed that a variety of agents could be used to stimulate differentiation of primary blast cells or immortalized cell lines isolated from leukemic patients. Since then the concept has been extensively studied and in some cases introduced into clinic [5,6]. *In vitro* investigations have demonstrated that all-*trans* retinoic acid (ATRA) can induce granulocytic differentiation in a number of myeloid leukemic cell lines [7]. To date the clinical usefullness of ATRA has been limited to patients with a relatively rare form of AML, acute promyelocytic leukemia (APL) in which a PML-RARα fusion protein is generated by a t(15;17)(q22;q12) chromosomal translocation. Differentiation therapy of APL using ATRA, especially in combination with arsenic trioxide, has significantly improved clinical prognosis [5,8]. Due to the limited success of ATRA, strenuous efforts have been made to identify alternative differentiating agents. 1,25-dihydroxyvitamin D_3 (1,25D) is another agent capable of inducing *in vitro* monocyte/macrophage differentiation of both normal and leukemic myeloid leukemic cell lines [3]. Despite having potent differentiation-inducing capacity against AML cell lines *in vitro*, 1,25D has not yet been shown to be effective in early clinical trials for the treatment of myelodysplastic syndrome (MDS) or AML [6]. One of the main reasons for the limited clinical utility of 1,25D is that at therapeutic concentrations this seco-steroid can induce potentially fatal systemic hypercalcemia [9]. Attempts to overcome this problem has focused on the synthesis of 1,25D analogs (VDAs) which retain differentiation-inducing capacity but lack the hypercalcemic effects [10,11]. Some of these compounds are used clinically in treatment of psoriasis

* Corresponding author. Tel.: +48 71 375 29 29; fax: +48 71 375 26 08.
E-mail address: ewa@protein.pl (E. Marcinkowska).

0145-2126/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.leukres.2009.10.004

[12], and secondary hyperparathyroidism [13]. Other VDAs have shown some potent anti-leukemic effects in animal models of leukemia [14–17] but these successes have not been carried over to the clinic [18–20].

Another key reason for the current lack of clinical success with non-calcemic VDAs could be due to inappropriate patient selection. AML is a heterogeneous disease and has been divided into various sub-groups depending on either morphology or cytogenetics. The most common classification system is the French–American–British (FAB) classification in which the AML is divided into eight subtypes (M0–M7), based on the type of cell from which the leukemia developed and on its degree of maturity [21]. However, limitations in the FAB system have led to the development of a new classification. In the World Health Organization (WHO) classification, AMLs are divided into four main groupings based on cytogenetic abnormalities, each group then contains numerous sub-categories [22]. This new system is more informative for hematologists than the FAB classification and has led to more successful 'patient-specific' treatments [1]. However, the FAB or WHO classifications have not been used routinely to predict susceptibility of blast cells from patients to 1,25D-induced differentiation.

In this study we compare the differentiation-inducing potential of 1,25D and five side-chain modified low calcemic VDAs [23,24] in a variety of myeloid leukemic cell lines representing different FAB classifications and in blast cells from patients with defined cytogenic abnormalities.

2. Materials and methods

2.1. Cell lines

HL60 and THP-1 cells were obtained from the European Collection of Cell Cultures. NB-4 and U937 cells were a kind gift from Dr. George P. Studzinski from University of Medicine and Dentistry of New Jersey. The cells were propagated as suspension cultures in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, Sigma, St. Louis, MO), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO). The cells were kept at standard cell culture conditions, i.e. humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The cell number and viability were determined by hemocytometer counts and trypan blue (0.4%) exclusion. For all experiments the cells were suspended in fresh medium containing 1,25D, VDA or the equivalent volume of ethanol as a vehicle control.

2.2. Isolation of mononuclear cells from patient's peripheral blood

Ten milliliter of peripheral blood was diluted with phosphate-buffered saline (PBS) in 1:1 ratio. Diluted blood was carefully layered onto the equal volume of LSM 1077 (PAA Laboratories GmbH, Pasching, Austria), and centrifuged at 400 × *g* for 30 min. The opaque interface containing the blast cells was transferred into fresh sterile tube, and washed three times with PBS. The cells were transferred to RPMI 1640 medium at the density of 10^6 cells/ml, supplemented with 10% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin and grown in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Due to the limited number of blast cells, the experiments on them were performed once.

The study population comprised of 32 patients diagnosed with AML. The study was accepted by the local Ethical Committee. The patients presented to the Department of Hematology, Blood Neoplasms and Bone Marrow Transplantation, Wrocław Medical University between October 2006 and May 2008 and gave informed consent for this study.

2.3. Chemicals and antibodies

1,25D and all VDAs were obtained from the Pharmaceutical Research Institute (Warsaw, Poland). The compounds were aliquoted and stored in glass ampoules under argon at −20 °C. Amount of the VDA in an ampoule was determined by UV spectrometry at 264 nm, compound was dissolved in an absolute ethanol to 100 μM, and subsequently diluted in the culture medium to the required concentration. The structures of VDAs used in this study are presented in Fig. 1, and methods of their synthesis were described earlier [18]. Calcium-phosphate regulating activities of all VDAs used in this study have been measured in mice and published before [15,16,19]. According to these publications the serum calcium levels in mice treated with all VDAs where comparable to control levels, and were significantly lower than calcium levels of animals treated with 1,25D.

MY4-RD1 (CD14) and MO1-FITC (CD11b) antibodies were purchased from Beckman Coulter (Fullerton, CA). Chemiluminescence Blotting Substrate was from Roche Diagnostics (Mannheim, Germany). Mouse monoclonal and rabbit polyclonal anti-VDR antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Goat anti-rabbit IgG and anti-mouse IgG conjugated to peroxidase were from Jackson ImmunoResearch (West Grove, PA). Other reagents were from Sigma (St. Louis, MO).

2.4. Determination of cell differentiation by flow cytometry

The expression of surface cell markers of monocytic differentiation CD14 and CD11b was determined by flow cytometry. Briefly, 1×10^6 cells were washed twice in PBS, then incubated with 0.5 μl MY4-RD1 (CD14) and 0.5 μl MO1-FITC (CD11b) on ice for 45 min. After the incubation, the cells were washed three times with ice-cold PBS and fixed in 1.5% paraformaldehyde. The cells were then suspended in 0.5 ml PBS and analyzed using FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). The acquisition parameters were set for an isotype control. Data analysis was performed with use of WinMDI 2.8 software (freeware by Joseph Trotter).

2.5. Preparation of cell lysates

Cell fractionation was performed as previously described [25]. The procedure was the same for cell lines and for blasts isolated from patient's blood. Briefly, cells (5×10^6) were washed three times with PBS, and lysed for 20 min on ice in 80 μl of lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100; pH 7.5) containing protease inhibitor cocktail (Roche Diagnostics). The lysates were separated by centrifugation for 5 min, at 14,000 rpm, at 4 °C. Supernatants were designated the cytoplasmic (C) fraction, and the nuclei remaining in pellets after one washing were sonicated for 10 s in the same volume of lysis buffer as before (80 μl/5×10^6 cells). Following sonication nuclei were centrifuged again for 5 min, at 14,000 rpm, at 4 °C and the final supernatants were designated the nuclear (N) fraction. Samples were denatured by adding 20 μl of 5× sample buffer and boiling for 5 min.

2.6. Western blotting of VDR

For western blotting 25 μl of cell lysates (derived from 1.25×10^6 cells) were separated on 12% SDS-PAGE gels and transferred to PVDF membranes. The membranes were dried, then blotted with primary antibody for 1 h at room temperature, washed three times with Tween-TBS and then blotted with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The protein bands were visualized with a chemiluminescence assay system. Then the membranes were stripped, dried again and probed with subsequent antibodies. Actin was probed as a control of equal loading and transfer of proteins.

2.7. Cytogenetic studies

Cytogenetic studies were performed using patient's bone marrow cells cultured in RPMI 1640 (unstimulated for 24 h and then stimulated with GM-CSF for 48 h). Cell harvesting and fixation were performed according to standard protocols. In most cases at least 20 metaphases were analyzed using standard GTG techniques. Clonal aberrations were defined according to the International System for Human Cytogenetic Nomenclature 2005 (ISCN2005). FISH technique with unique and wcp probes was used in order to verify the presence of aberrations of prognostic importance (poor risk: −5/5q−, −7/7q−, complex karyotypes, t(9;22), t(6;9) and good risk: t(8;21), t(15;17), t(16;16)). The analyses were performed using Applied Spectral Imaging Cytovision software (version 6.0.0.26) for chromosome and FISH analysis.

Cytogenetic studies performed in all 32 patients showed normal karyotypes in 12 and aberrant in 20 cases. Good risk aberrations typical of AML, such as t(8;21), t(15;17), t(16;16) were present in four patients, while poor risk aberrations, i.e. −5/5q−, −7/7q−, t(6;9), t(9;22), and complex karyotypes (with at least three aberrations) were present in 11 patients.

2.8. Statistical analysis

In order to analyze the results obtained in experiments with cell lines, Student's *t*-test for independent samples was used. To analyze patient's data distribution Shapiro–Wilk's test was used. In order to verify differences among groups ANOVA test was used for data with normal distribution and Kruskal–Wallis' test when data distribution could not be considered normal. Irrespective of the test used a p-value <0.05 was considered to be a significant difference.

3. Results

3.1. Differentiation-inducing activities of VDAs towards HL60, THP-1, NB-4 and U-937 cell lines

An 'ideal' VDA should be more potent than 1,25D in the induction of blast cell differentiation, but less potent in the regulation of