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Impaired nuclear localization of vitamin D receptor in leukemia cells resistant to calcitriol-induced differentiation

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Abstract

Calcitriol, the hormonal form of vitamin D_3, induces differentiation of monocytic leukemia cell lines in vitro, without inducing cytotoxicity of the cells. Besides this broad in vitro activity, a clinical implementation of calcitriol, or its analogs, as agents for differentiation therapy has been unsuccessful until now. A better understanding of cellular activities of calcitriol necessary for completion of cell differentiation program could help find better solutions for differentiation therapy of myeloid leukemias. In this paper we describe work carried on subline of acute monocytic leukemia, THP-1 resistant to calcitriol induced differentiation. This resistance correlates with impaired nuclear localization of vitamin D receptor, but not with its total expression in the cells. It also correlates with the resistance to calcitriol-induced growth inhibition, and to phorbol myristate acetate (PMA)-induced cell differentiation.

Keywords: Calcitriol; Differentiation; Proliferation; Vitamin D receptor; erk-1 and erk-2

1. Introduction

The main function of calcitriol (1,25-dihydroxyvitamin D_3) is regulation of calcium homeostasis of the organism, but the role of this compound in differentiation of various cells such as osteoclasts [1], keratinocytes [2] or monocytes [3] is now accepted. It is presently well documented that it also induces differentiation of leukemia cells from patients and leukemia cell lines in vitro [4].

The pleiotropic cellular effects of calcitriol are mediated by two different ways. The first one includes activation of vitamin D receptor (VDR) while, the second deals with activation of intracellular signaling pathways. The VDR belongs to the superfamily of nuclear steroid receptors, that act as ligand-inducible transcription factors [5]. The VDR is localized mainly in the cell nucleus, however a portion of the receptor resides in the cytosol where it colocalizes with endoplasmic reticulum, the Golgi complex and microtubules [6]. Liganded VDR associates with the specific DNA sequence named the vitamin D response element (VDRE). VDREs are localized in the promoter regions of calcitriol-regulated genes [5], but there are some genes regulated by calcitriol, without any known VDRE in their promoters.

Besides this transcriptional activity calcitriol stimulates some signal transduction pathways in the cells. For example, calcitriol activates Raf kinase [7], Src [8], mitogen activated protein kinases (MAPK) [9] and PI3-kinase [10] in target cells. Activation of intracellular signal transduction pathways is necessary for some of the biological activities of calcitriol. Inhibition of PI3-kinase pathway abrogates differentiation of HL-60 cells [11] and THP-1 cells [10]. Inhibition of different MAP kinase pathways has variable effects on the differentiation process, e.g. inhibition of erk-1 and erk-2 suppresses differentiation, while inhibition of p38 kinase enhances it [12]. Moreover, it has been shown that some analogs of calcitriol with greater cell-differentiation properties activate MAP kinases more robustly than calcitriol [13]. These observations suggest that rapid intracellular signaling is important for monocytic differentiation of leukemia cells, but its physiological importance and its relation to VDR-mediated signaling has not been elucidated. Involvement of intracellular kinases in the regulation of VDR activity by phosphorylation of VDR has been shown [14].

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For our studies on calcitriol-induced cell differentiation we used the human acute monocytic leukemia THP-1 cell line. Since THP-1 cell lines from two different sources were deposited at the Institute of Immunology and Experimental Therapy we wanted to compare their cell differentiation potential. Surprisingly one of the cell lines appeared to be resistant to calcitriol-induced differentiation and was named THP-1/DR (vitamin D resistant). We examined VDR expression in both cell lines. Both cell lines were found to express VDR, but the receptor is not localized in the cell nucleus in THP-1/DR cells. Resistance of these cells to calcitriol-induced differentiation was accompanied by their resistance to calcitriol-induced growth inhibition, and surprisingly to phorbol myristate acetate (PMA)-induced cell differentiation.

2. Materials and methods

2.1. Cell cultures

HL-60, THP-1 and THP-1/DR cell lines are available at the Institute of Immunology and Experimental Therapy where frozen stocks are maintained. HL-60 cells were propagated as a suspension culture in RPMI1640 medium supplemented with 8% fetal calf serum (FCS, Sigma Chemical Co., St. Louis, MO), 100 units/ml penicillin and 100 mg/ml streptomycin (both Polfa, Poland). THP-1 and THP-1/DR cells were cultured in RPMI 1640 supplemented with 4.5 g/l glucose, 0.05 mM 2-mercaptoethanol, 100 units/ml penicillin, 100 mg/ml streptomycin and 10% FCS. The cells were kept at standard cell culture conditions, i.e. humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.2. Antibodies

CD11b, CD14 and HLA-DR monoclonal antibodies, all FITC conjugated, were obtained from Sigma. Rabbit anti-VDR was from NeoMarkers (Fremont, CA) and rabbit anti-erk antibody and anti-phospho-erk antibody were from Cell Signalling Technology (Beverly, MA). Rabbit anti-histone H1 antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Goat anti-rabbit IgG conjugated to peroxidase was from Jackson ImmunoResearch (West Grove, PA).

2.3. Chemicals

Calcitriol was a kind gift from Dr. Pierre Weber from Hoffmann-la Roche S.A., Pharma Preclinical Research in Basel. Calcitriol was aliquoted and stored in glass ampoules under argon at −20 °C. Amount of compound in an ampoule was determined by UV spectrometry (Carl Zeiss spectrophotometer, Jena, Germany) at 264 nm. Prior to usage, the compound was dissolved in absolute ethanol at a concentration of 100 μM, and subsequently diluted in culture medium to the desired concentration.

Chemiluminescence blotting substrate was from Roche Diagnostics (Mannheim, Germany).

2.4. Cell differentiation assays

The cells were exposed to calcitriol or to phorbol myristate acetate (PMA) for 96 h. After completion of the exposure time the cells were collected, washed in phosphate-buffered saline (PBS) and counted in a hemocytometer.

To determine either CD11b, CD14 or HLA-DR expression by flow cytometry, 2×10^5 cells in 40 ml of PBS (supplemented with 0.1% BSA and 0.01% sodium azide) were mixed with 3 μl of monoclonal antibody solution (prechilled to 4 °C) and incubated on ice. The cell surface fluorescence was measured using FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). The damaged cells were labeled by adding 5 μl of propidium iodide solution (25 μg/ml) to each test tube just before the data aquisition. Data for damaged cells were not analyzed. Data analysis was performed with use of Cell Quest software (Becton Dickinson, San Jose, CA).

2.5. Preparation of total cell lysates

In order to obtain total cell lysates 3×10^6 cells were lysed in 80 μl of SDS-sample buffer for SDS–PAGE. Then samples were sonicated for 10 s twice in ice. The lysed samples were boiled and stored for subsequent electrophoretic analyses.

2.6. Isolation of cell nuclei

In order to isolate cell nuclei, 5×10^6 cells were lysed in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100 with protease inhibitor cocktail) for 30 min on ice. The cells were centrifuged for 10 min at 10,000 rpm at 4 °C, and the pellets were washed once again in lysis buffer. After next centrifugation the pellets were resuspended in 80 μl of SDS-sample buffer and sonicated for 10 s twice in ice. The lysed samples were boiled and stored for subsequent electrophoretic analyses.

2.7. Samples for erk activation

To study erk activation, the cells (1.5×10^6 per sample) were washed three times in serum-free RPMI and then starved for 3 h in fresh serum-free RPMI at 37 °C. Then the cells were exposed for 20 min to calcitriol or to 0.01% ethanol as a negative control (solvent for calcitriol). Then the cells were collected by centrifugation and lysed in 100 μl of SDS sample buffer, sonicated for 10 s and boiled.

2.8. Western blots

Equal volumes of samples (20 μl) were separated by SDS–PAGE and then transferred to PVDF membrane (Millipore, Billerica, MA). The quality of transfer was verified by staining the membrane with Ponceau S solution. For detection of VDR the membranes were probed with rabbit anti-VDR antibody. In the case of nuclear extracts, the same volumes of samples (20 μl) were separated in two parallel gels and then western blots for either VDR or histone H1 were performed. Membranes used for detection of MAP kinases were probed with either anti-total-erk or anti-phospho-erk antibody. All reactions were visualized with peroxidase-conjugated goat anti-rabbit antibodies and then by enhanced chemiluminescence system.

2.9. MTT assay

The cells were seeded in 96-well culture plates in 75 μl of medium. Next 75 μl aliquots of medium, containing calcitriol were added to the desired wells. Control wells were filled with the same volume of medium alone. The plates were incubated for 96 h under standard tissue culture conditions. For the last 3–4 h of incubation 20 μl of MTT solution were added to each well (stock solution: 5 mg/ml). At the end of 96 h incubation, 80 μl of lysing mixture were added to each well (lysing mixture: 225 ml DMF, 67.5 g SDS and 275 ml of distilled water). After 24 h in the dark at 37 °C, when formazan crystals were completely dissolved the plates were read on Multiscan RC Photometer (Labsystems, Helsinki, Finland) at 570 nm wavelength.

The OD represents cell viability and is expressed as a percent of control (cells grown on the same plate in the culture medium only). Mean cell viability was determined and plotted.

3. Results and discussion

In order to assess cell-differentiation potential the cells were exposed to calcitriol at concentrations of 10^{-6}, 10^{-7} and 10^{-8} M for 96 h. For comparison, we used HL-60 cells exposed to 10^{-8} M calcitriol for 96 h. Expression of CD11b and CD14 antigens was measured for quantitating differentiation. Table 1 shows expression of CD11b and CD14 in calcitriol-treated cells as compared to untreated cells. In THP-1 cells significant increase of CD11b and CD14 was detected, while there was no increase in CD11b and CD14 expression in THP-1/DR cells. Exposure to calcitriol resulted in relatively greater CD14 expression in HL-60 cells than in THP-1 cells. This was probably due to the fact that CD14 is detectable in untreated THP-1 cells while the antigen is not present on untreated HL-60 cells (not shown). Because cell differentiation is accompanied, or may be even preceded by cessation of proliferation, we wanted to determine if there is any difference in cell proliferation of THP-1 and THP-1/DR cells following calcitriol exposure. Since it has been shown previously, that HL-60 cells cease to proliferate in the presence of calcitriol [15], we used the HL-60 cells as a control for this experiment. Cell proliferation was determined by the MTT assay. Proliferation of cells treated with respective concentrations of calcitriol was presented as percent of vehicle-treated control. The results presented in the Table 2 show clearly that proliferation of THP-1/DR cells could not be inhibited by calcitriol (the results of calcitriol-treated samples did not differ significantly from the control samples). It must be pointed out that HL-60 cells were the most sensitive out of the cells studied to calcitriol-induced inhibition of proliferation. At every concentration of calcitriol used, inhibition of proliferation of HL-60 cells was more pronounced than that of THP-1 cells ($P < 0.01$).

We next wanted to determine if resistance of THP-1/DR cells could be caused by loss of vitamin D receptor in the cells. HL-60 cells have been reported to express VDR [16,17] and were therefore used as controls for VDR expression. Western blot analyses revealed that VDR is present in THP-1 cells as well as in THP-1/DR cells (Fig. 1, upper gel). Further analyses of isolated nuclei revealed that VDR expression was absent in the nuclear lysates prepared from THP-1/DR cells (Fig. 1, middle gel) but was clearly expressed in THP-1 nuclear lysates. Histone H1 served as a marker for authenticity of nuclear extracts from the cells

Table 1
Differentiation of THP-1, THP-1/DR and HL-60 cells induced by calcitriol

Cell line	Concentration (M)	CD11b (MC_{sam}/MC_{contr} ± S.E.M.)	CD14 (MC_{sam}/MC_{contr} ± S.E.M.)
THP-1	10^{-6}	6.04 ± 0.45	7.06 ± 1.30
	10^{-7}	4.79 ± 0.21	5.96 ± 0.83
	10^{-8}	3.60 ± 0.22	3.98 ± 0.07
THP-1/DR	10^{-6}	1.00 ± 0.05	0.97 ± 0.06
	10^{-7}	0.98 ± 0.10	1.02 ± 1.13
	10^{-8}	1.04 ± 0.08	1.11 ± 0.09
HL-60	10^{-8}	2.54 ± 0.27	9.03 ± 2.30

The cells were cultured for 96 h with calcitriol. Then the expression of CD11b and CD14 cell surface markers was determined in flow cytometry. Fold induction was calculated by dividing mean channel of fluorescence (MC) of the sample by MC of respective untreated control. Results of three separate experiments ± S.E.M. are presented.

Table 2
Inhibition of proliferation of HL-60, THP-1 and THP-1/DR cells in response to calcitriol

Concentration (M)	HL-60 (%)	THP-1 (%)	THP-1/DR (%)
10^{-6}	47.9 ± 3.2*	71.4 ± 3.6*	93.8 ± 1.7
10^{-7}	54.9 ± 2.6*	76.9 ± 1.6*	90.0 ± 1.7
10^{-8}	79.4 ± 1.5*	85.4 ± 1.5*	92.9 ± 1.8

The cells were cultured for 96 h with calcitriol. Cell proliferation was screened by MTT assay. Untreated cells were considered as 100%. The numbers represent viable cells in samples treated with calcitriol. Mean results ± S.E.M. are presented. An asterisk (*) indicates that the result differs significantly ($P < 0.01$) from untreated control.

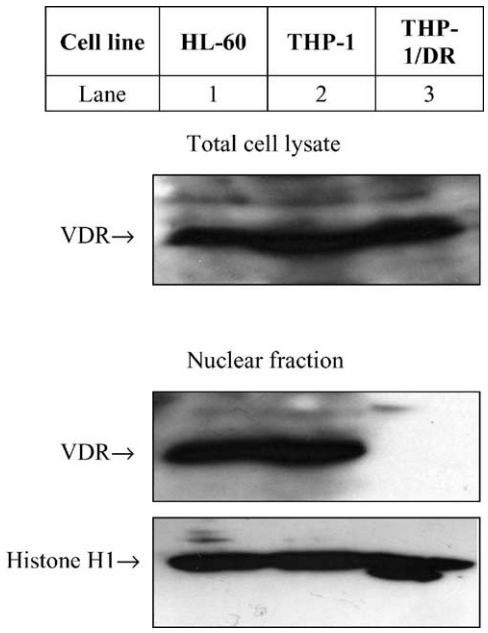

Fig. 1. Expression of VDR in HL-60, THP-1 and THP-1/DR cell lines. VDR was detected in lysates prepared from whole cells (upper gel), or from the cell nuclei (middle gel). The nuclear extracts were also probed for histone H1 (lower gel).

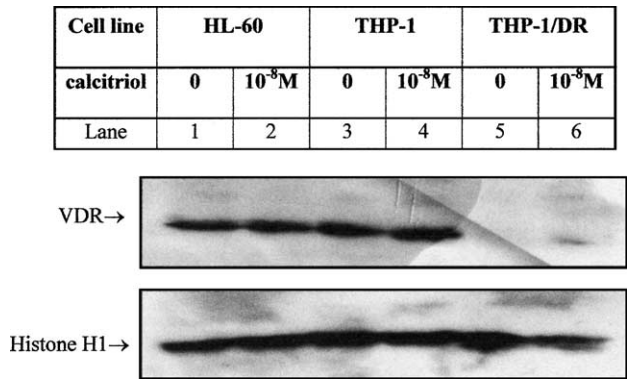

Fig. 2. Expression of VDR in the nuclei of the calcitriol-treated HL-60, THP-1 and THP-1/DR cells. The cells were either untreated (lanes 1, 3 and 5) or treated for 96 h with calcitriol at concentration of 10^{-8} M (lanes 2, 4 and 6). VDR was detected in lysates prepared from the cell nuclei (upper gel). The nuclear extracts were also probed for histone H1 (lower gel).

(Fig. 1, lower gel). VDR expression was detectable in nuclear extracts of calcitriol-treated HL-60 and THP-1 cells, but not in THP-1/DR cells treated with calcitriol (Fig. 2).

It has been reported previously that HL-60 and THP-1 cells differentiate in response to phorbol myristate acetate (PMA) [18,19]. Since cellular activity of PMA is very different from cellular activity of calcitriol, as PMA activates protein kinase C [18], we wanted to examine whether THP-1/DR cells were sensitive to PMA induced cell differentiation. As shown in the Table 3, the THP-1/DR appeared to be resistant to PMA-induced differentiation as compared to the THP-1 cells. There was significant ($P < 0.05$) increase of CD11b and CD14 antigen expression in THP-1 cells treated with 1 nM PMA for 96 h. This result suggests, that the cell differentiation pathway activated by PMA shares some commonality with the cell differentiation pathway activated by calcitriol although PMA and calcitriol do not induce differentiation in an identical manner. We observed significant increase in HLA-DR expression in PMA-treated THP-1 cells but not in calcitriol-treated THP-1 cells. As has been shown in the past the expression of HLA-DR increases in THP-1 cells in response to IFN-γ, but not in response to calcitriol [19]. PMA in turn upregulates the expression of IFN-γ receptor in THP-1 cells [20], what sensitizes these cells to IFN-γ.

Finally, we wanted to determine whether calcitriol treatment resulted in activation of erk-1 and erk-2 kinases in THP-1 and THP-1/DR cells, as it does in HL-60 cells. It should be noted that prior to stimulation with calcitriol, HL-60 cells should be serum-starved in order to study erk-1 and erk-2 kinase activation [9]. When the cells were stimulated with calcitriol in the presence of serum, activation of erk-1 and erk-2 by serum components obscured the effect of calcitriol (not shown). In THP-1 and THP-1/DR cells serum-starved for 3 h erk-1 and erk-2 were found to be still activated, while in HL-60 cells they were quiescent, as indicated by the lack of phosphorylation. When erk-1 and erk-2 were not phosphorylated in HL-60 control cells, they could be rapidly activated in response to calcitriol (Fig. 3). This effect was not observed in THP-1 and THP-1/DR cells in our experimental procedure. Surprisingly, our experiments revealed that the apparent molecular mass of erk-1, which should be 44 kDa, was higher in THP-1/DR cells than in either HL-60 cells or THP-1 cells. Thus it is tempting to speculate, that delayed migration of p44 in the gel is caused by its hyperphosphorylation. This does not appear to be mediated by mitogen-activated protein kinase kinase (MEK) since inhibition of MEK1 and MEK2 by a specific inhibitor

Table 3
Differentiation of THP-1 and THP-1/DR cells induced by PMA

Cell line	Concentration (M)	CD11b (MC_{sam}/MC_{contr} ± S.E.M.)	CD14 (MC_{sam}/MC_{contr} ± S.E.M.)	HLA-DR (MC_{sam}/MC_{contr} ± S.E.M.)
THP-1	10^{-9}	3.1 ± 0.5	2.1 ± 0.2	1.9 ± 0.1
THP-1/DR	10^{-9}	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.1

The cells were cultured for 96 h with 1 nM PMA. Then the expression of CD11b, CD14 and HLA-DR cell surface markers was determined in flow cytometry. Fold induction was calculated by dividing mean channel of fluorescence (MC) of the sample by MC of respective untreated control. Results of three separate experiments ± S.E.M. are presented.

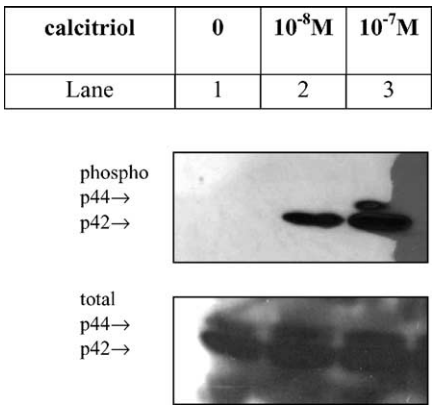

Fig. 3. Activation of erk-1 and erk-2 in serum-starved HL-60 cells. HL-60 cells were serum-starved for 3 h. Then the cells were exposed to calcitriol for 20 min at concentrations of 10^{-8} M (lane 2) and 10^{-7} M (lane 3). Control cells (lane 1) were treated with 0.01% ethanol (solvent for calcitriol). Then the cells were lysed and equal amounts of lysates were separated in SDS–PAGE. Phospho-erk-1 and erk-2 (upper gel) and total erk-1 and erk-2 (lower gel) were detected in Western blots.

PD98059 did not change the delayed migration of p44. PD98059 lowered the amount of active erk-1 and erk-2 in THP-1/DR cells (Fig. 4, lane 5). Therefore some constitutive modification of erk-1 in THP-1/DR cells could exist. As it has been reported previously that VDR interacts with activated PI3-kinase [10] it is possible that activated kinases such as PI3-kinase trap VDR in the cytosol of THP-1 cells.

Understanding the underlying causes of resistance to differentiation in cell culture models such as presented here for the THP-1/DR cells could help in understanding why

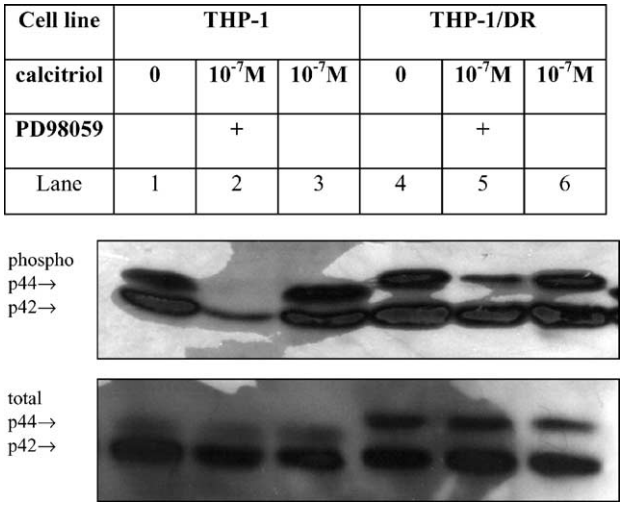

Fig. 4. Difference in molecular mass of erk-1 between THP-1 and THP-1/DR cells. The cells (1.5×10^6 per sample) were serum-starved for 3 h at 37 °C. For the last hour of incubation 20 μM PD98059 was added to one sample of each cells (lanes 2 and 5). Then the cells were for 20 min exposed to calcitriol at a concentration of 10^{-7} M (lanes 2, 3, 5 and 6). The control cells (lanes 1 and 4) were exposed to 0.01% ethanol (solvent for calcitriol). Phospho-erk-1 and erk-2 (upper gel) and total erk-1 and erk-2 (lower gel) were detected in Western blots.

differentiating therapy has very limited clinical applications [21]. Hematopoetic malignancies contain cells at different stages of differentiation, possibly with different sets of cumulated mutations. It is possible that differentiation therapy results in outgrowth of a proportion of the cells similar to THP-1/DR that do not respond to differentiation-inducing drugs. In such case a combination of differentially acting drugs could improve the final outcome of the therapy [21].

Our results further suggest that presence of VDR in the nuclei of target cells is of primary importance for calcitriol-induced cell differentiation. The activation of intracellular signaling pathways is of secondary importance and most probably amplifies the process of cell differentiation. This is in agreement with previous results showing that analogs of calcitriol with higher differentiation-inducing potential are more potent inducers of MAP kinase activation than calcitriol [13].

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