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Activation of intracellular signaling pathways is necessary for an increase in VDR expression and its nuclear translocation

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Abstract 1,25-Dihydroxyvitamin D_3 (1,25D) regulates gene transcription through the nuclear vitamin D receptor (VDR) and initiates rapid cellular responses via an unknown mechanism. Here we report that 1,25D induces a rapid increase in synthesis of VDR protein and its transport to the nucleus. These results are similarly obtained in myeloid leukemia cell lines, and in blast cells from blood of patients diagnosed with acute myeloid leukemia, subtypes M2 and M4. Our results suggest that stability of unliganded VDR is LY294002- and PD98059-dependent, and that ligation of VDR leads to its increased translation and nuclear translocation. The receptor localized in the cell nucleus is not exported back to the cytosol by exportin 1. We also show that the cytosolic portion of VDR in leukemia cells is localized in the vicinity of the plasma membrane, close to the F-actin cytoskeleton.

Keywords: Vitamin D; Nuclear receptor; VDR; Intracellular localization; Rapid responses; Differentiation; Acute myeloid leukemia

1. Introduction

1,25-Dihydroxyvitamin D_3 (1,25D) is a pleiotropic hormone, which regulates calcium homeostasis of the organism, induces differentiation and inhibits proliferation of various normal and cancer cells, including osteoclasts, keratinocytes and monocytes [1–3]. Even though the precise molecular basis of leukemia cell differentiation triggered by 1,25D is unclear, it is known that the major regulator of the biological activity of 1,25D is the nuclear vitamin D receptor (VDR) which can form homodimers [4], and the physiologically more active heterodimers with retinoid X receptor (RXR) [5,6]. Binding of the 1,25D enhances VDR/RXR heterodimerization and allows for the association with specific DNA sequence named the vitamin D response element (VDRE). The complex consisting of VDR/RXR and 1,25D binds to the VDRE and acts as a ligand-dependent transcription factor. This activated complex then recruits a coactivator complex, known as vitamin D receptor-interacting protein complex (DRIP) [7] and other proteins, histone acetylase among them. Acetylated histones relax chromatin structure to make DNA accessible and permit initiation of transcription of the target gene [8]. But it is also known that 1,25D activates some intracellular signaling pathways, so called ''rapid'' cellular responses [9]. It has been reported that these include the protein kinase C (PKC) pathway, calcium-dependent pathway, phosphatidylinositol 3-kinase (PI3-kinase) pathway and three groups of mitogen activated protein kinases (MAPKs) [10]. It is not clear at the moment if 1,25D-induced activation of the signal transduction pathways results in any modifications of VDR protein. It has not been well documented if VDR can be phosphorylated by Erk1,2 kinases, despite some data point at it [11]. Moreover, there are some data, which show that in response to 1,25D treatment VDR interacts with PI3-kinase and Src kinase [12–14]. The fact that activation of PI3-kinase is very important for 1,25D-induced differentiation has been known for some years [15], and it was later shown that 1,25D activates PI3-kinase [12] and its downstream element Akt kinase in the target cells [16,17]. Also activation of Src kinase has been documented in different cell line models [18,19]. How are activations of Src and PI3-kinase and their physical interactions with VDR connected is not known at the moment, but some lessons can be learnt from the estrogen receptor (ER) studies. Many biological effects of estrogens require activation of Src and PI3-kinases [20–22]. It has been documented that in breast cancer cells, which are responsive to estrogens, liganded ER forms stable ternary complex with Src kinase and with the regulatory domain of PI3-kinase, p85 [23]. Without liganded ER, the complex consisting of p85 and Src is unstable. Both ER and VDR belong to the superfamily of steroid receptors, which act as ligand dependent transcription factors. Thus in order to be active, these receptors must enter the cell nucleus. Intracellular localization of VDR has been an issue of some controversies since it is not fully agreed if VDR is translocated to the cell nucleus in response to 1,25D [24], or if it continuously shuttles between cytosol and nucleus and 1,25D traps it inside the cell nucleus [25]. It has been shown that the expression of VDR in the cells increases in response to 1,25D [26], however not in every type of target cells [27]. Thus in this study we wanted to find out if activation of PI3-kinase is important for expression and nuclear translocation of VDR in 1,25D-treated myeloid leukemia cells. For comparison we addressed

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Abbreviations: 1,25D, 1,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; RXR, retinoid X receptor; VDRE, vitamin D response element; DRIP, vitamin D receptor-interacting protein complex; PKC, protein kinase C; PI3-kinase, phosphatidylinositol 3-kinase; MAPK, mitogen activated protein kinase; ER, estrogen receptor; FCS, fetal calf serum; PBS, phosphate-buffered saline; KSR, kinase suppressor of Ras

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two MAPK pathways activated by 1,25D: Erk1,2 pathway and p38 pathway. In our studies we used two cell lines HL60 and THP-1. HL60 cells originate from acute myeloid leukemia subtype M2 [28], while THP-1 cells from acute myeloid leukemia subtype M5 [29] and both cell lines are responsive to 1,25D. In order to validate that the observations are not limited to the cell lines, we used in part of our studies blast cells isolated from blood of freshly diagnosed patients with acute myeloid leukemia.

2. Materials and methods

2.1. Cell lines

HL60 and THP-1 cells were obtained from the European Collection of Cell Cultures. These cells were propagated as a suspension culture 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 or the equivalent volume of ethanol as a vehicle control.

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

Ten milliliters of peripheral blood was diluted with phosphate-buffered saline (PBS) in 1:1 ratio. Diluted blood was carefully layered onto the equal volume of Histopaque-1077, and then was centrifuged at 400×*g* for 30 min. The opaque interface was carefully transferred into new, sterile tube, and washed three times with PBS. Then the cells were transferred to RPMI 1640 medium supplemented with 10% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin and kept at standard cell culture conditions.

2.3. Chemicals and antibodies

1,25D was a kind gift from Dr. Peter Weber (Hoffmann-La Roche S.A., Pharma Preclinical Research, Basel, Switzerland). PD98059 and LY294002 were from Calbiochem (San Diego, CA). Leptomycin B was from LC Laboratories (Boston, MA). Chemiluminescence Blotting Substrate was from Roche Diagnostics (Mannheim, Germany). Mouse monoclonal anti-VDR and rabbit polyclonal anti-ERα were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Goat anti-rabbit IgG and anti-mouse IgG conjugated to peroxidase or to RD1 were from Jackson ImmunoResearch (West Grove, PA). All other reagents were from Sigma (St. Louis, MO).

2.4. Cell fractionation

Cell fractionation was performed as previously described [29]. The cells were washed three times with PBS, and lysed for 20 min on ice in 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. The lysates were separated by centrifugation for 5 min, at 14000 rpm, at 4 °C. Supernatants were designated the cytoplasmatic fraction and the nuclei remaining in pellets after one washing were sonicated for 10 s in a new portion of lysis buffer. After sonication nuclei were centrifuged again and the final supernatants were designated the nuclear fraction.

In order to separate cytosol from membranes, the cells after treatment were washed three times with PBS and afterwards incubated in Hepes medium containing 20 μg/ml of digitonin and protease inhibitor cocktail for 5 min at room temperature and for 30 min on ice. After 5 min centrifugation at 3000 rpm, at 4 °C the supernatant was collected and designated the cytosolic fraction. The remaining pellets were lysed for 20 min on ice in lysis buffer containing protease inhibitor cocktail. The lysates were separated by centrifugation for 5 min, at 14000 rpm, at 4 °C. Here the supernatants were designated the membrane fraction and the nuclei remaining in pellets after one washing were sonicated for 10 s in a new portion of lysis buffer. After sonication nuclei were centrifuged again and the final supernatants were designated the nuclear fraction.

2.5. Western blotting

For Western blotting 25 μl of protein samples were separated on 12% SDS–PAGE gel and transferred to PVDF membranes. The membranes were dried, then blotted with primary antibodies for 1 hour at room temperature, washed three times with TBS and then blotted with horseradish peroxidase (HRP)-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. Each experimental condition was repeated at least twice.

2.6. Confocal microscopy

Coverslips were coated with poly-L-lysine solution just before the cells were seeded. The cells were treated as required and then they were fixed in 3% paraformaldehyde for 30 min at room temperature. The cells were then permeabilized with 0.1% Triton X-100 in PBS for 5 min. VDR was detected by incubating the samples with mouse anti-VDR (1:100) for 20 min. After three washes in PBS the samples were incubated with anti-mouse secondary antibodies conjugated to RD1 for 20 min and phalloidin conjugated to FITC and washed again. The specificity of staining was verified by omitting VDR staining in the procedure. Coverslips were finally mounted onto glass slides using Mowiol and the samples were analyzed with a Zeiss LSM 510 META confocal microscope (Thornwood, NY).

3. Results and discussion

3.1. Expression and nuclear translocation of VDR is an early response to 1,25D in leukemia cells

It has been shown in past that inhibitors of intracellular signaling pathways influence cell differentiation effect of 1,25D in myeloid leukemia cells. These observations have been described for HL60 and THP-1 cell lines. When inhibitors of MAPK/Erk1,2 signal transduction pathway and inhibitors of PI3-kinase have been added to the cells simultaneously with 1,25D, cell differentiation was markedly inhibited in comparison with the effect of 1,25D alone [12,15,30–33]. Since different MAPK pathways compose feedback loops in myeloid cells, inhibition of MAPK/p38 pathway resulted in an enhanced effect of 1,25D-induced cell differentiation [10,34]. An appearance of cell differentiation markers is a relatively late event in 1,25D initiated processes in leukemia cells, so we wanted to find an earlier effect of 1,25D. For this part of our experiments we applied cell fractionation method, which allows separation of cytosol and membranes from cell nuclei, in order to determine localization of VDR in further experiments. We observed that the increase in expression of VDR protein and its translocation to the cell nucleus could be detected as early as 1 h after treatment of the cells with 10 nM 1,25D. In untreated cells the intracellular level of VDR was relatively low, with little nuclear content (Fig. 1a). After 1 h of treatment the expression was higher and predominantly nuclear and it persisted until the late steps of cell differentiation (not shown here). It is noteworthy that similar upregulation and nuclear translocation of VDR could be observed in blast cells from myeloid leukemias in ex vivo cultures. In our experiments blast cells were isolated from blood of freshly diagnosed patients with acute myeloid leukemias, subtypes M2 and M4. The cells were exposed to 1,25D for 3 h and then the cells were collected and fractionated as above. As presented in Fig. 1b and c, nuclear content of VDR increased in comparison with control cells, however susceptibility of the cells to 1,25D differed between patients, and possibly was related to the subtype of acute myeloid leukemia.