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SYNERGISTIC DIFFERENTIATION OF U937 CELLS BY ALL-TRANS RETINOIC ACID AND 1α, 25-DIHYDROXYVITAMIN D3 IS ASSOCIATED WITH THE EXPRESSION OF RETINOID X RECEPTOR α

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Among the nuclear hormone receptors, the retinoid X receptors (RXRs) play a central role through their ability to heterodimerize with other members of this family of transcription factors, including retinoic acid (RA) and vitamin D (VD3) receptors. We have previously found that all-*trans* retinoic acid and 1α, 25-dihydroxyvitamin D3 cooperate to induce monocytic differentiation of U937 human leukemic cells. Here the expression of RXRα protein in myelomonocytic cells was studied by immunodetection using polyclonal antibodies. RXRα was detected upon exposure of cells to VD3 and higher levels were found in cells treated by combinations of RA and VD3 under conditions where both agents synergized for inducing monocytic properties.

The biologically active forms of vitamin D and vitamin A, 1α, 25-dihydroxyvitamin D3 (VD3) and all-*trans* retinoic acid (RA), are potent effectors of normal and leukemic myelomonocytic differentiation (1, 2, 3). They exert most of their action through nuclear signalling pathways by controlling the expression of target genes. Their nuclear receptors (VDR and RARs) belong to the same family of ligand-inducible transcriptional activators

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Abbreviations used : RA, all-*trans* retinoic acid; VD3, 1α, 25-dihydroxyvitamin D3; VD3R, Vitamin D3 receptor; RAR, all-trans retinoic acid receptor; RXR, 9-*cis* retinoic acid receptor; fMLP, formyl-methionine-leucine-proline peptide; PMA, phorbol myristyl acetate; NBT, nitro-blue tetrazolium; LPS, lipopolysaccharide.

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(4, 5). This protein family also includes retinoic X receptors (RXRs of α, β and γ types) (6, 7), a natural ligand of which is the 9-*cis* isomer of RA (8, 9). RARs and VDR require auxiliary factors for efficient binding to their responsive elements at the DNA level (10, 11, 12). By interacting with VDR and RARs as heterodimers, the RXR proteins facilitate high-affinity interactions with VD3 and RA responsive elements, thereby meeting the requirements for being auxiliary factors (7, 13, 14). The existence of these interactions suggests the possibility of interferences occuring between RA and VD3 signalling pathways. The human leukemia cell lines U937 and HL-60 provide experimental models for investigating such interactions. These cells possess VD3 and RA receptors (15, 16, 17). They respond to these agents by reduced proliferation and by the expression of a differentiated phenotype (reviewed in 18). Moreover, we found that RA, which by itself induces ill-defined phenotypic changes in U937 cells, strongly potentiates the action of VD3 in inducing their monocytic differentiation (19, 20). Cooperative effects have also been reported in HL-60 cells (21). In order to investigate a possible involvement of RXRα in these effects, we analyzed the expression of the RXRα protein by immunoblotting using a specific polyclonal antibody. The RXRα protein, which was barely detectable in proliferating non-differentiated cells, was largely increased following exposure of U937 and HL-60 cells to VD3. RA alone had no detectable effect. However, it increased RXRα expression in VD3-treated U937 cells, under conditions where a synergism between RA and VD3 was observed at the functional level.

MATERIALS AND METHODS

Cell culture and assessment of differentiation

U937 and HL-60 cell lines (American Type Culture Collection, USA) were routinely cultured at 37°C, 5% CO_2 in complete medium (RPMI 1640-10% fetal calf serum) (GIBCO BRL, France). The cell lines were tested for the presence of mycoplasma by analysis of the fluorescence of 4,6-diamino-2-phenylindole (DAPI), and viability (higher than 95%) was determined by trypan blue exclusion. Cells were incubated for various times with 100 nM VD3 (kindly provided by Hoffman-La Roche, Switzerland), without or with 100 nM RA (Sigma Chimie, France) (19). DNA synthesis was measured after incubation with 0.75 μCi [^{3}H]TdR (Dositeck, France) for 4 h in quadruplicate cultures (19) and results were expressed as the percentage of inhibition relative to control cells. Cellular respiratory burst activity was measured by luminol-enhanced chemiluminescence (using opsonized zymosan or fMLP as burst activators) (Sigma Chimie, France) and monitored over a fixed period in a luminometer (Lumicon, Hamilton, Switzerland) as previously described (20). For NBT reduction, 8 x 10^5 cells per ml were incubated for 30 mn at 37°C in complete medium containing 0,1 % NBT (Boehringer, Germany) and 100 ng/ml PMA (Sigma Chimie, France). The percentage of cells containing intracellular blue-black formazan deposits was then determined after fixation with 0,1% paraformaldehyde. At least 200 cells were assessed.

Detection of RXRα by western blotting

Whole cell and nuclear extracts were prepared as previously described by Rochette-Egly et al (22). The RXRα protein was immunodetected by using the rabbit polyclonal antiserum RPRXα(A). These antibodies were produced against a synthetic peptide corresponding to an amino acid sequence specific to mouse RXRα (region A). Proteins (70 µg) were fractionated by SDS-PAGE (10 % polyacrylamide) and then electroblotted onto nitrocellulose filters (NC) with a semi-dried electoblotter (Millipore, France) at 200 mA for 30 mn. The NC filters were then "blocked" in PBS-5% non fat powdered milk, incubated with polyclonal RPRXα(A) (diluted 1/300), extensively washed and immunoprobed with peroxydase labelled protein A (Amersham, USA). After extensive washing, specific complexes were revealed by chemiluminescence according to the manufacturer's protocol (Amersham, USA) (22, 23). Extracts from mouse RXRα-transfected COS-1 cells (mRXRα) were used as positive controls. The specificity of the bands was checked in inhibition experiments where RPRXα (A) antibodies were preincubated with the peptide used for immunization. The optical densities of the bands obtained on the films were determined by densitometric analysis with a Bioimage System (Millipore, France).

RESULTS

U937 cells loose their proliferative capacities and acquire functional properties upon RA and VD3 treatment (19, 20). As shown in Fig. 1, RA cooperates with VD3 in inhibiting cell growth, as assessed by the reduction of [^{3}H]TdR incorporation (Fig. 1A), and in increasing the number of differentiated cells, as determined by their ability to reduce NBT upon stimulation by phorbol esters (Fig. 1B). Measurements of luminol-enhanced luminescence emitted during oxidative burst were used to evaluate the intensities of the responses to opsonized particles of zymosan and to the chemotactic peptide fMLP (Fig. 1, C and D). The results illustrate the occurence of synergistic interactions between RA and VD3, leading to enhanced phagocytosis of zymosan particles (Fig. 1C) and inducing a response to fMLP (Fig. 1D), after 48 h treatment. This sensitivity to fMLP is not acquired upon treatment with only one of the two agents.

Nuclear extracts from U937 cells were processed for immunoblotting by using the rabbit polyclonal antibodies specific for RXRα, together with peroxidase labelled protein A (Fig. 2). A unique band, with the same electrophoretic mobility as the recombinant RXRα protein produced by transfected COS-1 cells, was detected in U937 cells cultured for 48 h in the presence of 100 nM VD3 or 100 nM RA plus 100 nM VD3 (Fig. 2, lanes 3, 4 and 5). This band was not detectable in extracts from untreated proliferating cells or from cells treated by 100 nM RA alone (Fig. 2, lanes 1 and 2). Minor bands above the recombinant RXRα protein were observed (lane 5) when high quantities of protein were loaded on the gel. Time-course studies were performed on whole cell extracts, as illustrated in Fig. 3A, and the levels of

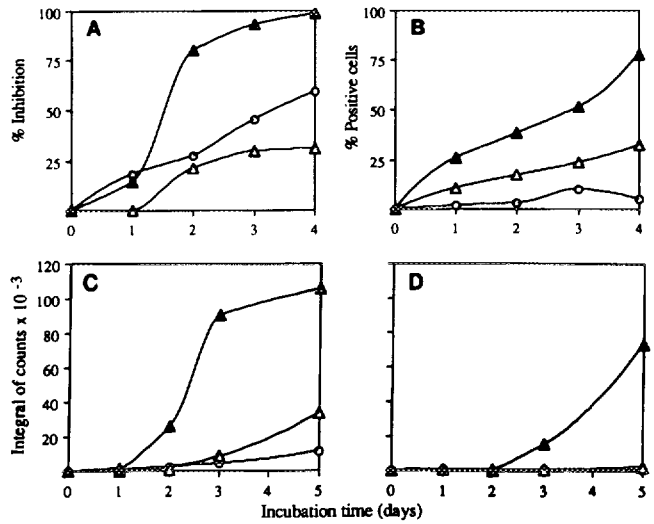

Figure 1.
Cell growth inhibition (A) and % of NBT-positive cells (B) and expression of chemiluminescence triggered by phagocytosis of opsonized zymosan (C) or by exposure to fMLP (D). U937 cell differentiation was induced by 100 nM RA (-O-), 100 nM VD3 (-△-) or 100 nM RA + 100 nM VD3 (-▲-), during the indicated times. In A, DNA synthesis of cells (2.5 x 10^4 per ml) was measured as described in Materials and Methods. Results are expressed as the % of inhibition of [^{3}H]TdR incorporation relative to undifferentiated cells, and each point represents the mean of 3 experiments with quadruplicate wells per point. NBT reduction (B) was performed as described in Materials and Methods and results are expressed as the % of cells containing intracellular blue-black formazan deposits. In C and D, the chemiluminescence of 10^5 cells was assayed as described in reference 19. Undifferentiated cells did not significantly respond to zymosan or fMLP (data not shown). Results are expressed in integrals of counts. In B, C, D, results and are mean of 3 independent experiments differing by less than 10%.

immunodetected protein were estimated by image processing of the films in a series of independent experiments (Fig. 3B). We could not ascertain the presence of the RXRα protein in non-differentiated cells, thus corroborating the results obtained on nuclear extracts. The protein began to be observed after 24 h treatment in extracts of cells treated by VD3 alone. Maximal expression was achieved at 48h treatment and decreased thereafter. In RA plus VD3- treated cells, the protein began to be detected after 12 h treatment. A large but transitory increase was observed at 48 h, the immunodetected band being 4-fold more intense than in cells treated by VD3 alone (Fig. 3A, compare lane 10 to lane 4, and Fig. 3B). The electrophoretic mobilities of the bands obtained in VD-treated cells was slightly different from that in COS-transfected cells. This difference could arise from a limited proteolysis of the receptor in whole cell extracts, since macrophages have high levels of endogenous proteases. This difference was not observed in the case of nuclear extracts (Fig. 2).

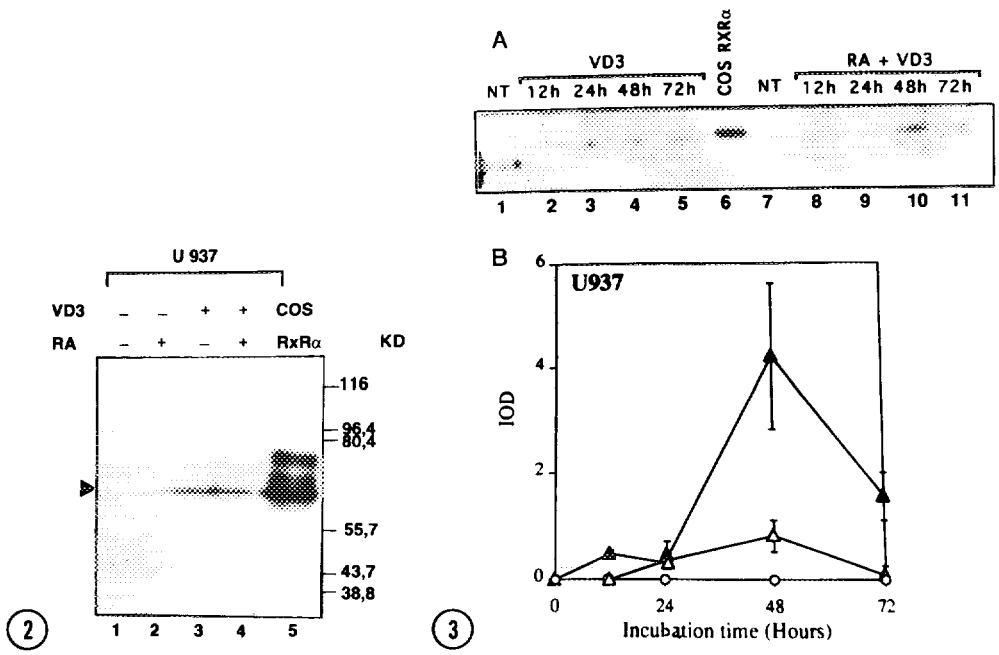

Figure 2.
Characterization of RXRα in U937 cells. Cells were grown for 48 h without (lane 1) or with 100 nM RA (lane 2), 100 nM VD3 (lane 3), or 100 nM RA + 100 nM VD3 (lane 4). Nuclear extracts (70 µg protein) of U937 cells were fractionated by SDS-PAGE, electrotransferred to NC filters and immunoprobed with polyclonal RPRXα(A) together with peroxidase-labelled protein A. The specific complexes were revealed by chemiluminescence. As positive control, extracts (2 µg) of mRXRα-transfected COS-1 cells were run in parallel (lane 5). Prior to immunodetection, the filters were stained with Ponceau S to check the uniformity of samples amounts (data not shown).

Figure 3.
Time-course of RXRα induction in U937 cells. In A, cells were grown in the absence (lanes 1 and 7) or in the presence of 100 nM VD3 (lanes 2-5) or of RA + VD3 (100 nM each) (lanes 8-11), for 12 h (lanes 2 and 8), 24 h (lanes 3 and 9), 48 h (lanes 4 and 10) and 72 h (lanes 5 and 11). Whole cell extracts (70 µg) were fractionated by SDS-PAGE, electrotransferred onto NC filters, stained with Ponceau S and immunoprobed with RPRXα(A) together with peroxidase-labelled protein A. Extracts (0.5 µg) of mRXRα-transfected COS-1 cells were run in parallel (lane 6). In B, the signals revealed by chemiluminescence on western blots were quantified using the Integrals of the Optical Densities (IOD) program of a Millipore Bioimage System. U937 cells were treated by 100 nM R A (-O-), 100 nM VD3 (-△-) or 100 nM RA + 100 nM VD3 (-▲-) for the indicated times. Each point represents the mean +/- SD of three independent experiments. For 12 h points, the error bars are smaller than the symbol.

HL-60 cells undergo monocytic differentiation upon exposure to VD3 and granulocytic differentiation when treated by RA. When treating cells with a combination of RA and VD3 (or VD3 derivatives), synergistic or additive effects are obtained, depending on the type of observation carried out, as cell

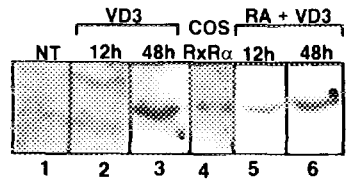

Figure 4.
Time-course of RXRα induction in HL-60 cells. Cells were either non treated (lane 1), treated with VD3 (100 nM) (lanes 2 and 3) or with RA + VD3 (100 nM each) (lanes 5 and 6), for 12 h (lanes 2 and 5) or for 48 h (lanes 3 and 6). Extracts (0.5 µg) of mRXRα-transfected COS-1 cells were run in parallel (lane 4). Experimental conditions were the same as in Fig. 3.

proliferation or some parameters of cell differentiation (20, 21). Here we found that, as in U937 cells, the RXRα protein could not be detected in proliferating HL-60 cells and that it was expressed upon incubation with VD3 (Fig. 4, lane 3) but not with RA (data not illustrated). When combining both agents, RXRα levels were detected after 12 h treatment (Fig. 4, lane 5). However, contrarily to U937 cells, HL-60 cells exposed for 48h to RA plus VD3 did not express larger amounts of RXRα protein (Fig. 4, compare lane 3 to lane 6).

DISCUSSION

In spite of the high sensitivity of enhanced chemiluminescence detection methods used to reveal immunoblots, we could not ascertain the presence of RXRα in extracts from HL-60 and U937 proliferating cells. The fact that dividing cells express very small amounts, if any, of RXRα protein raises the question of its role as an auxilliary factor for RARs and VD3R at the onset of the cascade of events triggered by RA or VD3. It must be determined whether very low levels of RXRα are sufficient to activate a limited set of target genes involved in the first steps of the responses or alternatively, if RA and VD3 receptors may combine at this stage with other nuclear factors. The presence of RXRβ needs to be investigated (13). The formation of homodimers or RAR/VD3R heterodimers should also deserve attention (24).

In both cell lines, RXRα could be detected after 12 h exposure to combinations of both agents, while remaining practically undetectable in cells treated by each agent alone. We and others have found that RA and VD3 cooperate in inhibiting HL-60 and U937 cell proliferation (19, 20, 21). Taken together, these observations might indicate an involvement of RXRα in the induction of cell growth arrest.

The highest levels of RXRα were observed after 48 h treatment, either with VD3 alone in the case of HL-60 cells, or with combinations of RA and VD3 in U937 cells. They were associated with the expression of monocytic properties. Besides this temporal correlation, a relationship was found between

the intensities of the responses of HL-60 and U937 cells exposed to combinations of RA and VD3 (19) and the expression of RXRα. It is well known that VD3 induces monocytic differentiation in both cell lines, while RA alone has ill-defined effects on U937 phenotype and induces a granulocytic phenotype in HL-60 cells (2, 18). We have previously found that acting on U937 cells, RA synergized with VD3 in inducing phagocytic properties, sensitivity to fMLP and release of IL-6 upon LPS stimulation, while on HL-60 cells, the effects of RA on VD3-induced differentiation were either only additive (phagocytosis of zymosan), practically undetectable (sensitivity to fMLP) or even partially antagonistic (IL-6 production) (20). Here we found that RA synergized with VD3 to enhance RXRα expression in U937 but not in HL-60 cells. This parallelism between phenotypic changes and RXRα induction suggests that RXRα might be required for VD3-induced expression of monocytic specific genes. Other data are in agreement with this hypothesis. They are provided by studies on PMLRAR, an abnormal protein generated by a t(15;17) chromosomal translocation in acute promyelocytic leukemia (26). PMLRAR is indeed able to sequester RXR, thus preventing VDR-induced reporter gene expression (27). Acting probably through this mechanisms, it was shown to inhibit VD3-induced differentiation when expressed in transfected U937 cells (28).

Our data, showing wide variations of RXRα protein within 3 days of treatment, indicate that its expression is tightly regulated. To our knowledge, there are no published data about the promoter of RXRα gene. Therefore, we do not know if RXRα gene includes RAR or VDR response elements that would account for a direct regulation of gene expression. At the mRNA level, studies on various rat tissues did not reveal any effect of VD3 or retinoids on RXR expression (29). However, increased RXRα gene expression was observed in RA-treated F9 teratocarcinoma cells (30). RXRα transcripts have been detected by Northern-blotting in non-differentiated HL-60 cells (27). By reverse transcription-polymerase chain reaction assays, RXRα transcripts were also detected in dividing U937 cells and were not increased upon VD3 treatments (T. Commes and D. Parienté, personnal observation). The presence of mRNA in HL-60 and U937 cells at a time when the protein could not be detected suggests the existence of regulatory mechanisms operating at posttranscriptional levels. Pulse-chase labelling experiments have shown that VDR stability was increased upon association of the protein with its ligand (31, 32). This VD3-induced up-regulation might in turn influence RXRα levels since the protein could be stabilized in VDR/RXR heterodimers. The mechanisms through which RA increased RXRα expression in RA plus VD3-treated cells is still obscure. Since the natural ligand of RXR which might increase its stability is 9-*cis* RA, it should be determined whether it is produced in cell cultures by isomerization of the all-*trans* isomer (33).

Whatever the mechanisms involved in RXRα expression, further studies are required to determine whether this increase in RXRα is merely a side effect or whether RXRα plays a central role in mediating the action of RA and VD3.

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