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Effects of 1,25-Dihydroxy Vitamin D3 on All-Trans Retinoic Acid Sensitive and Resistant Acute Promyelocytic Leukemia Cells

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Following challenge of the acute promyelocytic leukemia (APL) cell line, NB4, with 1, 25 dihydroxy vitamin D3 (1, 25 D3), no increase in the expression of the monocytic surface markers, CD11c, CD14 and HLA-DR is observed. By contrast, 1, 25 D3 increases the expression of CD11b, an early myeloid marker and enhances adherence to plastic following priming of the cells with phorbol 12-myristate 13-acetate (PMA). NB4.306 and NB4.007/6, two all-trans retinoic acid-resistant cell lines originated from NB4 promyelocytes and lacking expression of a complete form of PML-RAR, are totally resistant to 1, 25 D3-dependent induction of CD11b. In addition, NB4.306 cells do not show enhanced plastic adherence following treatment with the vitamin D metabolite and PMA. NB4 and NB4.306 express similar amounts of the transcripts coding for the vitamin D3 receptor and the retinoid accessory receptors, RXRα and RXRβ, both in basal conditions and upon treatment with 1, 25 D3.

The NB4 acute promyelocytic leukemia (APL) cell line recapitulates the most characteristic features of the APL blast. It expresses the PML-RAR fusion protein derived from the typical t(15:17) balanced chromosomal translocation and it is exquisitely sensitive to the cyto-differentiating action of all-trans retinoic acid (ATRA) and its synthetic derivatives (1-3). PML-RAR is an aberrant protein which results from the juxtaposition of the transcription factor PML with the major isoform of the retinoic acid receptor (RARα) present in the hematopoietic system (4). PML-RAR is believed to play a dual role in the homeostasis of the APL blast. On the one hand, the protein seems to be responsible for the differentiation block at the level of the promyelocyte observed in this type of leukemia (2). On the other hand and somehow paradoxically, PML-RAR is thought to be instrumental in conferring selective ATRA-sensitivity upon the APL blast (2). PML-RAR-dependent inhibition of maturation along the myelogenous pathway leading to the granulocyte has been suggested to result from a sequestration of RXRs into transcriptionally inactive complexes (2, 5). RXRs are retinoic acid accessory receptors which form heterodimers with RARα and all the other RAR isoforms, allowing high affinity binding of the heterodimeric complexes on the corresponding binding sites present on retinoid-regulated genes (6). RXRs act as heterodimeric partners not only for RARs but also for other members of the nuclear receptor superfamily (7-9). In particular, RXRs are known to heterodimerize with the 1,25 dihydroxy vitamin D3 (1,25 D3) receptor (VDR) (7). 1, 25 D3, is a potent inducer of monocytic differentiantion in normal and leukemic myeloid cells (10, 11) and non-hypercalcemic derivatives of 1, 25 D3 are potentially useful cyto-differentiating agents. In this report, we evaluate the ability of the NB4 cell line to respond to 1, 25 D3 in terms of cyto-differentiation. In addition, we compare the 1,25 D3 responsiveness of NB4

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cells to that of NB4.306 and NB4.007/6, two recently isolated ATRA-resistant NB4 sublines which do not express the complete form of the PML-RAR protein (12, 13).

MATERIALS AND METHODS

Cell culture conditions and reagents. The PML-RAR positive acute promyelocytic leukemia NB4 cell line (1) was a kind gift of Dr. M. Lanotte (Unite' INSERM 301, Paris, France). The ATRA-resistant NB4-derived sublines, NB4.306 and NB4.007/6, were described in previous reports (12, 13). The PML-RAR negative acute promyelocytic leukemia cell line, HL-60, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). NB4 and HL-60 cells were routinely passaged in RPMI 1640 containing 10% FCS (GIBCO-BRL, Gaithersburg, MD), whereas NB4.306 and NB4.007/6 were cultured in the same medium containing ATRA (10^{-6} M). NB4.306 and NB4.007/6 cells were cultured for four days in medium devoid of ATRA before the beginning of each experiment. ATRA and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO), whereas 1, 25 D3 was a kind gift of Dr. M. Uskokovic (Hoffmann LaRoche, Nutley, NJ). Stock solutions of ATRA (10^{-2} M) and PMA (1.5×10^{-3} M) were prepared by dissolving the compounds in DMSO. 1, 25 D3 stock solutions were prepared by dissolving the compound in ethanol at 10^{-3} M.

Analysis of cyto-differentiation markers. The myeloid surface markers, CD11b, CD11c, CD14 and HLA-DR were determined by flow cytometry, as already described (3). Specific phycoerythrin-conjugated monoclonal antibodies and relative negative controls were purchased from Beckton-Dickinson, Mountain View, CA. The ability of cells to reduce nitroblue tetrazolium (NBT) was evaluated spectrophotometrically according to the method of Pick et al. (14).

Northern blot analysis and polymerase chain reaction (PCR) amplification. Total RNA was prepared from NB4 and NB4.306 cells and used for Northern blot analysis as already described (3). The probes utilized for Northern blot analysis were a full-length human RXRα cDNA (15) and a full-length human glucose-6-phosphate-dehydrogenase (G6PD) cDNA (16). PCR amplifications of the VDR, RXRα, RXRβ and β-actin transcripts were carried out from total RNA after reverse transcription using the gene AMP kit (Cetus Perkin Elmer, Norwalk, CT) according to the instructions of the manufacturer. The cDNAs were amplified with the following couples of amplimers: 5′TCATTGCCATACTGCTGGAC3′ (nucleotides 473-492), 5′AGGACTCATTGGAGCGCAAC3′ (complementary to nucleotides 877-896) for VDR (17); 5′CTCAATGGCGTCCTCAAGGT3′ (nucleotides 412-431), 5′TTCAGCCCCATGTTTGCCTC3′ (complementary to nucleotides 680-699) for RXRα (15); 5′AACTCAACAGTGTCACTCCC3′ (nucleotides 604-623), 5′TCTTCTGTTCCACAGCAAGC3′ (complementary to nucleotides 1042-1061) for RXRβ (9); 5′GCGCTCGTCGTCGACAACGG3′ (nucleotides 60-79), 5′GATAGCAACGTACATGGCTG3′, (complementary to nucleotides 430-449) for β-actin (18). Samples were subjected to 30 cycles of amplification (94°C for 1 min, 55°C for 2 min and 72°C for 3 min). Amplifications were carried out simultaneously on one fourth of the same reverse transcription reaction. PCR was performed in conditions of linearity in terms of RNA concentration and during the logarithmic phase of the amplification by Taq polymerase. Amplified products were visualized by ethidium bromide staining following electrophoresis on 1.5% agarose gel.

RESULTS

The ability of myeloid cells to reduce NBT is associated with maturation along both the granulocytic and monocytic lineage. As shown in Fig. 1, NB4 cells are sensitive to the cyto-differentiating action of pharmacological concentrations of ATRA, whereas the NB4.306 subline is resistant to the effects of the retinoid. In fact, upon treatment of the two cell lines with ATRA (10^{-6} M) for 4 days, a clear increase in the ability of NB4 cells to reduce NBT is evident, whereas a similar effect is not observed in the case of NB4.306. Upon treatment with 1,25 D3 (10^{-7} M) for the same length of time, neither NB4 nor NB4.306 cells show an increase in NBT-reducing activity.

To investigate whether NB4 cells and the ATRA-resistant clone are completely unresponsive to the effects of 1,25 D3, the expression of a series of myeloid differentiation markers was studied. For this purpose, we chose CD11b, a marker expressed in both the granulocytic and monocytic lineage, as well as CD14, HLA-DR and CD11c, three surface antigens expressed predominantly in the monocyte. As shown in Table 1, in basal conditions, the four markers are practically absent from the plasma membrane of both NB4 and NB4.306 cells. The level of expression of CD14, HLA-DR and CD11c is left unaltered in either NB4 or NB4.306 cells incubated for 4 days in the presence of 1,25 D3 (10^{-7} M). The compound causes a sustained increase in the number of CD11b-positive NB4 cells and in the amount of CD11b cell associated fluorescence. Similar effects are not observed in the ATRA-resistant NB4.306 subline. Treat-

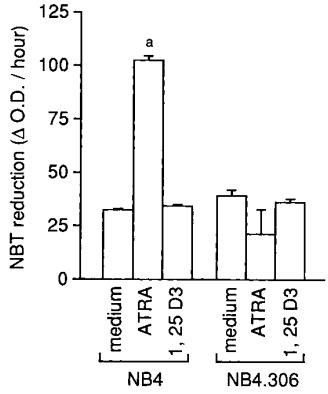

FIG. 1. Effects of ATRA and 1, 25 D3 on the NBT-reducing activity of NB4 and NB4.306 cells. NB4 and NB4.306 cells (4×10^5/ml) were incubated for 4 days with medium alone and medium containing ATRA (10^{-6} M) or 1, 25 D3 (10^{-7} M). Cells were harvested and processed for the determination of NBT reducing activity. The results are normalized for the number of cells in each sample and expressed as NBT reducing activity (△ O.D. at 540 nm/hour). Each value is the mean ± S.D. of three determinations. [a]Significantly higher relative to the corresponding medium treated group according to the Tukey's test ($p<0.01$). A representative example of two independent experiments is shown.

ment of NB4 cells for 4 days with ATRA results in a marked increase in CD11b positivity. ATRA treatment of NB4 cells is also associated with a significant elevation in the surface expression of the two monocytic markers CD11c and HLA-DR. CD11b, CD11c and HLA-DR expression is not changed in NB4.306 promyelocytes. Resistance of NB4.306 cells to the effects of 1,25 D3 and ATRA is not the consequence of a generalized block in the granulocytic and monocytic maturation program, since these cells are as sensitive as the parental cell line in responding to the monocyte-macrophage differentiation agent PMA. In fact, following a 4-day-incubation-period with the phorbol ester derivative (2×10^{-7} M), CD11b and CD11c surface expression is similarly increased in both NB4 and NB4.306 cells.

TABLE 1
Effects of 1,25 D3, ATRA and PMA on the Expression of Myeloid Cell Surface Markers in NB4 and NB4.306 Cell Lines

		% positive cells (CAMF)				
		Neg	CD11b	CD14	HLA-DR	CD11c
NB4	Medium	1 (20)	8 (28)	1 (21)	1 (23)	1 (23)
	1,25 D3	1 (24)	36 (56)	4 (30)	2 (27)	4 (27)
	ATRA	1 (22)	79 (99)	1 (24)	13 (26)	34 (30)
	PMA	1 (23)	48 (52)	1 (22)	1 (24)	43 (53)
NB4.306	Medium	1 (31)	6 (33)	1 (22)	1 (24)	1 (23)
	1,25 D3	1 (25)	4 (32)	1 (26)	1 (24)	2 (26)
	ATRA	1 (20)	5 (34)	1 (24)	1 (26)	4 (28)
	PMA	1 (20)	55 (56)	1 (24)	5 (33)	39 (45)

Note. NB4 or NB4.306 cells were seeded at 4×10^5/ml and treated with medium alone (medium) and medium containing 1,25 D3 (10^{-7} M), ATRA (10^{-6} M) or PMA (2×10^{-7} M) for 4 days. Following treatment, cells were harvested and processed for the flow cytometric analysis of the indicated surface markers. Neg = cells incubated with an irrelevant antibody for the determination of background fluorescence. CAMF = cell associated mean fluorescence in arbitrary units. The values shown are representative of two independent experiments.

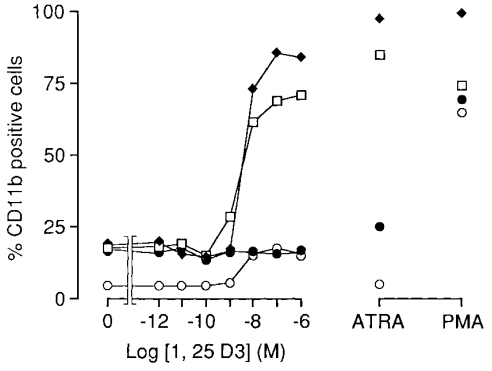

FIG. 2. Effects of 1, 25 D3, ATRA and PMA on the expression of CD11b in NB4, HL-60, NB4.306 and NB4.007/6 cells. NB4 (solid diamonds), HL-60 (open squares), NB4.306 (solid circles) and NB4.007/6 (open circles) cells (4×10^5/ml) were incubated for 4 days with medium alone and medium containing ATRA (10^{-6} M), PMA (2×10^{-7} M) or the indicated concentrations of 1, 25 D3. Cells were harvested and processed for the determination of the percentage of CD11b positive cells by flow cytometry. A representative example of two independent experiments is shown.

Using CD11b as a differentiation marker, we examined the relative sensitivity of various APL cell lines to 1,25 D3, by determining dose-response curves. As shown in Fig. 2, a dramatic increase in CD11b-positive NB4 cells is observed starting from a 1,25 D3 concentration of 10^{-8} M. Maximal induction of CD11b expression is observed at 10^{-7} and maintained at 10^{-6} M. The 1,25 D3 dose-response curve for the induction of CD11b in NB4 cells is not different from that observed in HL-60, an APL-derived and PML-RAR negative cell line, which is fully responsive to the cyto-differentiating effects of both ATRA and the vitamin D3 active metabolite (19). At all the concentration used, NB4.306 is totally unresponsive to the action of 1,25 D3 in terms of CD11b induction. Similarly, NB4.007/6 (13), another independently derived ATRA-resistant NB4 subline, does not show expression of CD11b upon treatment with 1, 25 D3. As expected, NB4, NB4.306, NB4.007/6 and HL-60 respond to PMA with a dramatic increase in the levels of CD11b, whereas, upon treatment with ATRA, the expression of the surface marker is augmented only in NB4 and HL-60 cells.

Treatment of NB4 promyelocytes with PMA is known to cause an increase in cell adherence to plastic surfaces, which can be enhanced by addition of 1, 25 D3 to the growth medium (20). As illustrated in Fig. 3, only a very small proportion of NB4 cells incubated for 80 hours with medium alone or medium containing 1, 25 D3 (2×10^{-7} M) is capable of adhering to plastic. If cells are treated for the same amount of time with PMA (2×10^{-7} M), a remarkable increase in the number of adherent cells is evident. Relative to what is observed with PMA alone, an almost 3 fold enhancement in adhesion is observed if cells are treated with the combination between the phorbol ester and 1,25 D3. In the presence of PMA, NB4.306 cells show an increase in plastic adherence of the same magnitude as that observed in NB4 cells. However increased adherence is not further augmented by treatment with the combination between PMA and 1, 25 D3.

As a first step in understanding the molecular mechanisms underlying the cross-resistance of ATRA-resistant NB4 sublines to 1, 25 D3, we studied the level of expression of the mRNAs coding for VDR and its heterodimerization partners, RXRα and RXRβ. Northern blot experiments demonstrated that VDR, RXRα and RXRβ transcripts of the expected molecular weight (approximately 4.8, 5.5 and 2.8, and 2.4 kb, respectively) are expressed in approximately equal amounts in NB4 and NB4.306 cells (data not shown). As shown in Fig. 4A, RT-PCR experiments performed with amplimers specific for the three receptor mRNAs support the idea

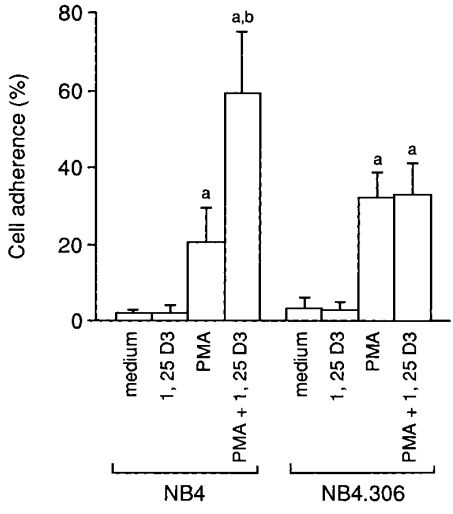

FIG. 3. Effects of 1, 25 D3, PMA and the combinations of the two compounds on the adherence to plastic of NB4 and NB4.306 cells. NB4 and NB4.306 cells (4×10^5/ml) were incubated for 80 hours with medium alone and medium containing 1, 25 D3 (2×10^{-7} M), PMA (2×10^{-7} M) or the combination of the two compounds. Adherence was assessed as the percentage of total cells in each well adhering to plastic. The results are the mean ± S.D. of three independent experiments with each experimental point run in triplicate. [a]Significantly higher relative to medium according to the Tukey's test ($p<0.01$). [b]Significantly higher relative to 1, 25 D3 and PMA according to the Tukey's test ($p<0.01$).

that NB4 and NB4.306 do not differ significantly in their ability to express VDR, RXRα and RXRβ mRNAs in basal conditions. In addition, it is evident that treatment of both cell lines with 1,25 D3 for 4 days does not significantly affect the level of expression of the three receptors. Challenge of NB4 cells with ATRA for 4 days causes a dramatic downregulation of the RXRα transcript, whereas it does not significantly alter the level of expression of RXRβ or VDR mRNAs. Expression of β-actin mRNA, a transcript expressed constitutively in many cell types, is left unaffected by treatment with either ATRA or 1, 25 D3. To confirm the effects of 1, 25 D3 and ATRA on RXRα in NB4 and NB4.306 cells in a more quantitative way, the

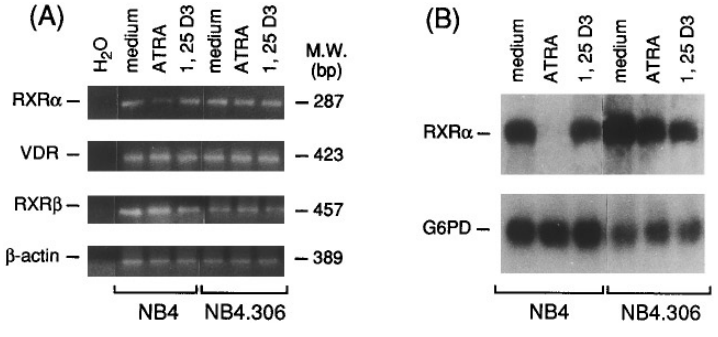

FIG. 4. Effects of 1, 25 D3 and ATRA on the expression of VDR, RXRα and RXRβ mRNAs in NB4 and NB4.306 cells. NB4 and NB4.306 cells (4×10^5/ml) were incubated for 4 days with medium alone and medium containing ATRA (10^{-6} M) or 1, 25 D3 (10^{-7} M). Cells were harvested and processed for the extraction of total RNA and subsequent RT-PCR (panel A) or Northern blot analysis (panel B). The length of each PCR amplified cDNA band is indicated on the right. Northern blot analysis was performed with radiolabeled probes corresponding to RXRα and G6PD cDNAs. The same filter was sequentially hybridized with RXRα and G6PD probes.

Northern blot experiment shown in Fig. 4B was performed. Whereas 1, 25 D3 has no effect on RXRα mRNA expression, treatment of NB4 cells with ATRA for 4 days causes a dramatic downregulation of the transcript. In NB4.306 cells, following treatment with either ATRA or 1, 25 D3, expression of the RXRα transcript is not altered relative to basal conditions. Expression of the G6PD mRNA is similar in all the experimental conditions, supporting the specificity of the effects observed at the level of the transcript coding for RXRα.

DISCUSSION

The biological effects of 1, 25 D3 are believed to be mediated mainly by intranuclear receptors (VDRs) belonging to the same family of steroid and retinoid receptors (21), although receptor-independent effects have also been reported (22). VDRs are ligand-activated transcriptional factors which interact with their cognate responsive elements (VDRE) on vitamin D regulated genes either as homodimers or as heterodimers with RXRs (23, 24). In any case, VDR-RXR heterodimers bind with much higher affinity than VDR homodimers to VDREs (24) and are thought to be the physiologically relevant form of the transcriptionally active complex.

APL cells are characterized by a specific chromosomal rearrangement involving RARα, which leads to the expression of an abnormal fusion protein known as PML-RAR. PML-RAR is believed to sequester RXRs into transcriptionally inactive complexes, preventing these accessory receptors from interacting with cognate heterodimerization partners, including VDR (23). This should lead to complete or partial unresponsiveness to 1, 25 D3. In line with this concept, overexpression of PML-RAR in U937 myeloid cells leads to resistance to the vitamin D active metabolite (25). Concordantly, the results presented in this paper demonstrate that PML-RAR expressing NB4 cells are refractory to the induction, by 1, 25 D3, of monocyte differentiation markers, like CD14, CD11c and HLA-DR. In addition, treatment of NB4 promyelocytes with 1, 25 D3 does not result in an increase in the ability of these cells to reduce NBT. However, NB4 cells are sensitive to the action of 1, 25 D3, in terms of CD11b surface expression. In fact, induction of CD11b by 1, 25 D3 shows a dose-response curve that is not different from that observed in HL-60 cells, a PML-RAR-negative promyelocytic cell line which is fully sensitive to the cyto-differentiating action of the vitamin D3 metabolite (19). Furthermore, as already reported (20), NB4 cells increase 1, 25 D3-dependent adherence to plastic, in the presence of PMA. This suggests that PML-RAR expression in its natural cellular context does not necessarily inhibit all the aspects of 1, 25 D3-induced cyto-differentiation.

NB4.306 is an ATRA-resistant NB4 subline characterized by the presence of a PML-RAR transcript which is not different in size from the corresponding mRNA observed in the parental NB4 cell line (12). However, NB4.306 cells do not express significant amounts of a functional PML-RAR fusion protein which can be recognized by antibodies specific to the F domain of RARα (12, 26). Our data demonstrate that NB4.306 is completely resistant to the cyto-differentiating effects of 1, 25 D3. In addition, the same resistance is observed in NB4.007/6, another ATRA-resistant NB4 cell line, which does not synthesize detectable levels of PML-RAR immunoreactive protein. At present, we do not have an explanation for the cross-resistance to 1, 25 D3 of ATRA-resistant APL cells, although it is clear that the two phenotypes cosegregate in this biological system and are possibly mediated by common mechanisms. Cross-resistance does not seem to arise from a defect in the expression or the regulation of VDR and RXRs. In fact, although we did not directly measure the levels of the three types of receptors, the corresponding mRNAs are equally expressed in both NB4 and NB4.306 cells and 1, 25 D3 does not significantly affect the levels of the three transcripts. Based on the results obtained in the NB4 cell line and its ATRA-resistant sublines, we suggest that VDR-RXR heterodimer-independent mechanisms underlie the response of NB4 cells to 1, 25 D3.

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