RAPID COMMUNICATION

Retinoic Acid Is Required for and Potentiates Differentiation of Acute Promyelocytic Leukemia Cells by Nonretinoid Agents

By Alex Chen, Jonathan D. Licht, Yu Wu, Nella Hellinger, William Scher, and Samuel Waxman

Patients with acute promyelocytic leukemia (APL) associated with the (t(15;17) translocation and fusion of the promyelocytic leukemia (PML) and retinoic acid receptor-α (RAR-α) genes achieve complete remission but not cure with all-trans retinoic acid (RA). NB4, a cell line derived from a patient with t(15;17) APL that undergoes granulocytic differentiation when treated with pharmacologic doses of RA, was used as a model for differentiation therapy of APL. We found that NB4 cells are resistant to differentiation by nonretinoid inducers such as hexamethylene bisacetamide (HMBA), butyrates, vitamin D₃, or hypoxanthine, all of which can induce differentiation in the commonly used HL60 leukemia cell line. Preexposure of NB4 cells to low concentrations of RA for as short as 30 minutes abolished resistance to nonretinoids and potentiated differentiation. Sequential RA and HMBA treatment yielded maximal differentiation when treated with pharmacologic doses of RA, to RA may occur after protracted treatment. In analogy with combination chemotherapy, combination of lower doses of differentiation-inducing drugs may offer the potential to prevent or overcome the resistance encountered in single-agent therapy. Therefore, in an attempt to improve the therapeutic outcome of single-agent RA in the treatment of APL, we combined RA and nonretinoid differentiation inducers in a model system. The human APL cell line NB4, which contains the (t(15;17) translocation characteristic of APL, and expresses the promyelocytic leukemia (PML)-retinoic acid receptor-α (RARα) fusion gene, was used in these studies. We confirmed that NB4 cell differentiation was induced by RA but found these cells to be resistant to a wide variety of nonretinoid differentiation inducers. Remarkably, however, the NB4 cells became exquisitely sensitive to differentiation by RA but found these cells to be resistant to a wide variety of nonretinoid differentiation inducers. These data support a novel strategy of combining differentiating agents in the treatment of APL.

MATERIALS AND METHODS

Cell culture. NB4 cells (kindly provided by Dr M. Lanotte, Hopital St Louis, Paris, France) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Hyclone, UT), 1 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. These cells were obtained in October 1991, treated for mycoplasma with BM cycline (Boeringher-Mannheim, Indianapolis, IN), and maintained in continuous culture. The present studies were initiated in July 1993 and karyotypic analysis showed no major chromosomal differences from the NB4 cell line obtained in 1991. The cells contain one copy of (t(15;17) with two partially deleted chromosomes 17 detected in some cells. The PML-RARα fusion gene was shown in both the 1991 and 1993 cultures by reverse transcription-polymerase chain reaction (RT-PCR) analysis and the described immunophenotype, as determined by fluorescence-activated cell sorting (FACS) analysis, remained unchanged (data not shown). HL-60 cells (kindly provided by Dr T. Breitman, National Cancer Institute [NCI], Bethesda, MD) were cultured as described above except that the medium was supplemented with an additional
RESULTS

NB4 cells are resistant to differentiation by nonretinoid agents. RA, 13-cis RA, and 9-cis RA induced differentiation of about 49% to 55% of NB4 cells after 5 days of treatment as determined by NBT positivity (Table 1). The induction of differentiation was time and concentration dependent, with up to 85% of NB4 cells NBT-positive (NBT+) after exposure to 1 to 2 μmol/L RA for 6 days (data not shown). Whereas HL-60 cells can be induced to differentiate by multiple nonretinoid agents,24 the NB4 cell line was found to be resistant to differentiation by the same agents (Table 1). Higher concentrations of compounds such as HMBA (5 to 10 mmol/L) or sodium butyrate (5 mmol/L) also failed to induce differentiation of NB4 cells despite causing loss of cell proliferation and the promotion of cell death (data not shown). Despite similar inhibition of cell growth in both HL-60 and NB4 cells, Ara-C induced differentiation in HL-60 but not NB4 cells.

RA pretreatment of NB4 cells potentiates the rapid onset of differentiation by nonretinoid inducers. NB4 cells were incubated with or without RA for 24 hours, washed in fresh medium, and then treated for 2 days with nonretinoid agents known to induce HL-60 cell differentiation (Table 2). RA (0.5 μmol/L) alone was a very weak inducer of differentiation at 48 hours (2.5% NBT+) and 72 hours (5% NBT+). In striking contrast, when either HMBA or sodium butyrate was added to cells pretreated with RA, there was a marked potentiation of differentiation with greater than 50% of the cells becoming NBT+ within 48 hours (Table 2). RA pretreatment also stimulated differentiation induced by D3 and Ara-C, although these combinations were significantly less potent. Pretreatment with 9-cis RA, which can either bind directly to RARs,35 or 13-cis RA, which can be isomerized to retinoids that bind RARs also potentiated differentiation by nonretinoid inducers. In contrast, arctinoid, an antiproliferative retinoid that does not bind to nuclear RARs,6 did not stimulate differentiation (data not shown). This suggests that RA- and RAR-directed gene transcription may be required for the nonretinoid agents to mediate differentiation.

The time course of differentiation induced by HMBA after RA pretreatment over a 5-day period is shown in Fig 1A. Treatment of NB4 cells with RA (0.5 μmol/L) alone led to a slow induction of differentiation with 10% NBT+ cells by 3 days with a maximal effect of 55% NBT+ by 6 days. HMBA treatment alone was completely ineffective in the induction of differentiation. Sequential treatment with 24 hours of RA followed by HMBA resulted in faster induction of differentiation. Under these conditions, NBT+ cells were observed after 1 day of HMBA treatment and maximal induction (~85% NBT+ cells) occurred by the third day. Simultaneous treatment with the two agents also resulted in 85% NBT+ cells (data not shown). RA pretreatment for 24 hours followed by 3 days of culture in drug-free media led to only minimal differentiation (<10% NBT+ cells). The sequence of treatment was crucial for differentiation. When

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### Table 1. Induction of Differentiation by Single Agents in NB4 and HL-60 Cell Lines

<table>
<thead>
<tr>
<th>Drug</th>
<th>%NBT*</th>
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<tbody>
<tr>
<td></td>
<td>NB4</td>
</tr>
<tr>
<td>All-trans RA (1 μmol/L)</td>
<td>49</td>
</tr>
<tr>
<td>13-cis RA (2 μmol/L)</td>
<td>55</td>
</tr>
<tr>
<td>9-cis RA (1 μmol/L)</td>
<td>51</td>
</tr>
<tr>
<td>HMBA (2 mmol/L)</td>
<td>7</td>
</tr>
<tr>
<td>DMSO (1.25%)</td>
<td>4</td>
</tr>
<tr>
<td>Sodium butyrate (1 mmol/L)</td>
<td>1</td>
</tr>
<tr>
<td>Cytosine arabinoside (30 μmol/L)</td>
<td>2</td>
</tr>
<tr>
<td>Hypoxarhine (2 mmol/L)</td>
<td>4</td>
</tr>
</tbody>
</table>

Cells were treated with inducers for 5 days at the concentrations indicated. Differentiation was assessed by reduction of NBT.

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### Table 2. Potentiation of Differentiation by RA Followed by Nonretinoid Differentiation Inducers

<table>
<thead>
<tr>
<th>Drug</th>
<th>%NBT*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h Pretreatment Without RA</td>
</tr>
<tr>
<td>All-trans RA (0.5 μmol/L)</td>
<td>2.5</td>
</tr>
<tr>
<td>HMBA (2 mmol/L)</td>
<td>1.0</td>
</tr>
<tr>
<td>Sodium butyrate (1 mmol/L)</td>
<td>0.3</td>
</tr>
<tr>
<td>Vitamin D3 (0.1 μmol/L)</td>
<td>0.0</td>
</tr>
<tr>
<td>Cytosine arabinoside (30 μmol/L)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

NB4 cells were incubated for 24 hours with or without RA (0.5 μmol/L), washed, and cultured for 2 additional days in complete medium supplemented with differentiation inducers. There were no significant differences in the final cell densities obtained within each pair of experiments with or without 24-hour RA pretreatment.
NB4 cells were preincubated with HMBA for 24 hours and then treated with RA for 3 days, there was no stimulation of differentiation over that achieved by RA alone (data not shown).

RA treatment at low concentrations of brief duration potentiates differentiation. The level of HMBA-mediated differentiation stimulated by RA pretreatment was dose-dependent and reached a maximum between 0.5 μmol/L and 1 μmol/L RA (Fig 2). Levels of RA lower than typically used in vitro and in vivo still had a marked effect on differentiation. A concentration of 50 mmol/L RA followed by HMBA yielded a level of differentiation equal to that obtained after a transient 24-hour exposure to 1 μmol/L RA as a single agent (Fig 2). RA was not continuously required during the pretreatment phase because only 30 minutes of RA pretreatment was sufficient for maximal stimulation of HMBA-induced differentiation (Fig 3). To examine the need for new protein synthesis during the pretreatment phase, cells were incubated with cyclohexamide (10 μg/mL) and RA for 30 minutes, then washed and treated with HMBA for 3 days.

This treatment resulted in a 70% inhibition of cell growth yet produced only an ~20% decrease in the level of differentiation (data not shown).

RA/HMBA treatment decreases proliferative capacity of NB4 cells after differentiation induction. There was similar inhibition of cell growth by RA (0.5 μmol/L) followed by HMBA for 3 days compared with continuous RA exposure or RA for 24 hours followed by drug-free treatment (Figs 1B and 2). Therefore, the potentiation of differentiation by the sequential treatment at 3 days was observed before a decrease in cell density resulting from growth inhibition. Cultures containing terminally differentiated cells are expected to contain decreased cell number without an increase in trypan blue-positive cells. Transient 24-hour exposure to RA alone increased the level of NBT-cells in a concentration-dependent manner; however, after 5 days of culture only the highest concentration of RA used (5 μmol/L) led to a decrease in the total cell number (Fig 2). At this concentration of RA only 25% of the cells became NBT-cells. In contrast, sequential 1 μmol/L RA pretreatment followed by incubation with HMBA treatment led to maximal differentiation (85% NBT-cells) and was associated with a 50% decrease in the total cell number (Fig 2). Similarly, prolonged treatment with HMBA (>3 days) after RA (0.5 μmol/L) pretreatment was associated with a decrease in cell number without loss of viability (Fig 1B).

RA potentiates differentiation by clinically useful butyrate derivatives. Sodium butyrate and other butyrate derivatives or pro-drugs including phenylbutyrate and phenylacetate did not induce differentiation of NB4 cells at any of the concentrations studied (1 to 5 mmol/L) (Table 1 and data not shown). Pretreatment with 0.5 μmol/L RA for 3 hours followed by a 3- or 4-day treatment with the butyrate derivatives led to a potentiation of differentiation that was concentration dependent (Fig 4). At an equimolar concentration of the derivatives, sodium butyrate was the most potent of the three, followed by phenylacetate and phenylbutyrate. As in the case of HMBA, sequential RA/butyrate treatment resulted in potentiation of differentiation before a decrease in total cell number which occurs at the end of 4 days without an increase in trypan blue-positive cells (Fig 4).

Sequential RA and HMBA treatment potentiates differentiation of primary cultures of human APL cells. Mononuclear cells were obtained from the peripheral blood or bone marrow of three patients with APL. Patient 1 had previously shown clinical resistance to RA and leukemic cells from this patient failed to respond to RA when cultured in vitro (Fig 5). However, when these resistant cells were sequentially treated with RA and HMBA, there was a 10-fold increase in NBT-cells (Fig 5). Patient 2 was in relapse after complete remission induced by RA 1 year earlier and patient 3 was newly diagnosed and not previously treated with RA. Leukemic cells from patients 2 and 3 did respond to RA when cultured in vitro (15% to 20% NBT-cells) (Fig 5). Still, sequential RA/HMBA treatment of leukemic cells from these patients led to a threefold increase in NBT-cells compared to continuous treatment with RA. As a result, 40% to 50% of the cells were NBT-cells. In all cases the sequence of exposure to RA followed by HMBA appeared to induce more differentiation than simultaneous exposure to RA and HMBA. None of the patients' leukemic cells responded to HMBA alone.
Fig 2. RA potentiates differentiation by HMBA. NB4 cells were preincubated for 24 hours with the indicated dose of RA, washed, and cultured for 3 additional days in the presence of 2 mmol/L HMBA (—○—) or no drug (—□—).

After 6 days in culture, the untreated primary leukemic cells were predominantly promyelocytes and blasts (Fig 6A). RA as a single agent induced the appearance of cells with decreased dense granularity but few mature myeloid cells (Fig 6B). In contrast, primary cultures sequentially treated with RA and HMBA displayed maturing myeloid cells including metamyelocytes, band forms, and some segmented granulocytes (Fig 6C). By FACS analysis differentiation by RA/HMBA compared with RA alone was manifested by a 2.5-fold increase in the proportion of large, homogenous, less granular and more mature cells and a doubling of the intensity of expression of CD15, a marker of mature myeloid cells. Similar in NB4 cells, sequential treatment was associated with a doubling of the proportion of more mature cells and a twofold increase in the proportion of cells expressing CD15 (data not shown). No significant change in cell number was observed in APL cells cultured for 6 days with or without treatment and cell viability remained greater than 90%.

DISCUSSION

We showed that myeloid differentiation of the NB4 APL cell line can be induced by pharmacologic concentrations of retinoids but not by a variety of nonretinoid inducers. Not only was this resistance reversed by pretreatment with RA, but the sequential combination of RA and HMBA or butyrates led to synergistic induction of differentiation and terminal differentiation. In contrast, other myeloid cell lines such...
Fig 5. RA pretreatment potentiates HMBA-induced differentiation of primary culture of mononuclear cells from APL patients. Non-adherent mononuclear cells were isolated from the peripheral blood or bone marrow of three APL patients. The cells were pretreated with or without RA (0.5 μmol/L) for 24 hours, washed in culture media, and grown in the presence or absence of RA (0.5 μmol/L), HMBA (2 mmol/L), or both RA and HMBA for 5 days. Differentiation was assayed by NBT reduction and viability by trypan blue exclusion. Untreated cells (■); RA continuous (□); RA/HMBA-simultaneous treatment (△); RA/HMBA-sequential treatment (●); HMBA (▲).

as HL-60 can be induced to differentiate with both RA and all of the nonretinoid inducers. To date, RA has only shown consistent beneficial clinical results in the treatment of APL. Both the unusual RA sensitivity of APL and its insensitivity to nonretinoids may be related to the characteristic chromosomal translocation t(15;17) and the resultant PML-RARα fusion gene. The PML-RARα fusion protein is believed to act in a dominant negative manner, interfering with normal RA-mediated gene transcription and differentiation in APL cells. In support of this model, the DNA-binding specificity and the retinoic-acid responsiveness of the PML-RARα fusion protein is altered from that of wild-type RARα. In addition, expression of the PML-RARα protein can inhibit RA-dependent transcriptional activation by wild-type RARα. In U937 and HL-60 model systems, expression of the PML-RARα protein suppressed differentiation and programmed cell death when cells were cultured with physiologic concentrations of RA (0.001 μmol/L), yet enhanced differentiation when cultured with pharmacologic doses of RA (1 μmol/L).

Fig 6. RA and HMBA treatment of primary leukemic cell cultures yields more mature granulocytic cells. Leukemic cells from patient 3 were cultured for 5 days in the presence or absence of inducers. The cells were spun onto a microscope slide, fixed, and stained with Wright-Giemsa. Cells were visualized on a Zeiss-Axiophot microscope (Thornwood, NY) at 80× magnification. (A) No treatment; (B) RA (0.5 μmol/L); (C) RA (0.5 μmol/L) for 24 hours, washed, and followed by HMBA (2 mmol/L).
The PML-RARα fusion protein may also explain the resistance of APL cells to differentiation with D3. U937 cells expressing PML-RARα fail to differentiate in response to D3 unless pretreated with RA.43 The vitamin D receptor (VDR) binds to DNA as a heterodimer with the retinoid X receptor (RXR) and activates transcription through D3 response elements.44-47 In vitro experiments suggest that the PML-RARα fusion protein competes with VDR for limiting amounts of RXR and prevents VDR binding to D3 elements.42 Upon treatment with RA the PML-RARα fusion protein undergoes relocation within the nucleus, which may allow RXR to become available for use by other nuclear receptors.29 Because nonretinoids such as dimethyl sulfoxide (DMSO) and butyrate do not bind to RARs, another more indirect mechanism must be in place to prevent their action.

Although nonretinoids alone failed to induce differentiation of APL cells, sequential treatment of NB4 cells with RA/HMBA led to superinduction of differentiation (>85% NBT+ cells) by 3 days after initiation of HMBA. In the absence of HMBA, differentiation of greater than 50% of the cell population required continued exposure to RA for 5 to 6 days. New protein synthesis was not required for RA-mediated potentiation of nonretinoid-induced differentiation because cycloheximide did not significantly block the effect. In addition, arotinoid, which does not bind to the RARs, failed to potentiate differentiation. Therefore, it appears that RA acts either through pre-existing PML-RARα fusion protein or wild-type RARs in the NB4 cell to reverse the insensitivity of NB4 cells to nonretinoid inducers and stimulate differentiation. Remarkably, only a 30-minute pretreatment with RA led to maximal potentiation of differentiation by HMBA. Despite washing, the exogenously added RA could be retained within the cell, bound to the RARs or cytosolic retinoid acid-binding proteins, and yield transcriptional or other effects for a prolonged period.

Potentiation of differentiation of NB4 cells by the combination of RA and nonretinoid inducers could be shown before inhibition of cell growth. RA, followed by more than 3 days of exposure to nonretinoid inducers, but not RA alone, led to a significant, RA dose-dependent decrease in total cell number without a loss of cell viability. This indicates that the RA/HMBA or RA/butyrate treatments yielded more terminally differentiated cells, decreasing their proliferative capacity. Although 20% of the APL cells in primary culture treated with RA became NBT+, there was only modest evidence of morphologic differentiation. In contrast, when treated sequentially with RA and HMBA, a greater number of primary APL cells became NBT+, developing into more mature cells of the granulocytic lineage. Although RA/HMBA (>3 days) treatment had an antiproliferative effect on NB4 cells, the total number of primary leukemic cells did not increase while in culture and did not decrease with treatment. This may indicate that a higher proportion of fresh leukemic cells have withdrawn from the cell cycle relative to the NB4 cell line that was selected for its ability to grow in vitro. This, in turn, suggests that more prolonged treatment with RA/HMBA may be required to observe antiproliferative effects in patients than in the model cell line.

Prior studies in the HL60 model system40,42 as well as in fresh leukemic cells41 showed that RA could cooperate with a nonretinoid inducer such as HMBA to yield synergistic induction of differentiation. This suggests the presence of separate pathways for the induction of hematopoietic differentiation by RA and nonretinoid inducers. However, the complete insensitivity of NB4 cells to nonretinoid inducers, relieved by RA, suggests that the two pathways may be linked. In support of this, preliminary data indicate that 3 days of exposure to HMBA enhances RARα expression in NB4 and fresh APL monoclonal cells.31 This effect is additive with the level of activation seen by RA alone. This suggests that HMBA and RA may activate an overlapping set of genes that may influence myeloid differentiation. HMBA-induced differentiation of hematopoietic cells is also associated with activation of PKC activity,49 downregulation of the c-myc and c-myb genes,50 decreased levels of cyclin A and cyclin-dependent kinases (CDK),51 and the accumulation of unphosphorylated form of the retinoblastoma protein.52,53 The result is a prolongation of the G1 phase of the cell cycle, a time when cells become committed to differentiation. In this context, untreated APL cells could be intrinsically blocked at one or more of the steps required for cellular differentiation. Treatment with RA would then make the cell competent to respond to a secondary signal delivered by agents such as HMBA, butyrate, and D3.

Although RA treatment induces clinical remission in about 90% of patients with APL it does not eradicate the malignant clone because the chimeric PML-RARα transcript can still be detected by PCR.52,53 Continuous use of RA is associated with clinical resistance. This may be caused by increased catabolism or RA, or the induction of cytosolic RA-binding protein that may sequester RA in the cytoplasm preventing its intranuclear activity.54,55 RA-resistant APL cell lines have also been generated in vitro associated with alteration in the function or loss of expression of the PML-RARα fusion protein.56-58 The occurrence of these mutations emphasizes the necessity to devise clinical approaches that minimize the duration of patient exposure to RA while maximizing response. Previous in vitro studies showed that pretreatment of leukemia cells with chemotherapeutic agents followed by differentiation inducers such as RA or HMBA results in enhanced differentiation, terminal cell division, and programmed cell death.59-61 Clinically, combining chemotherapy with RA is associated with a longer disease-free interval and, in several cases, disappearance of the malignant clone as determined by PCR analysis. However, there are still frequent reports of relapse after this approach.56

In the NB4 cell line, brief treatments with low concentrations of RA followed by nonretinoid inducers yielded more extensive and rapid differentiation than RA alone. Sequential treatment with RA and HMBA had a similar effect in primary cultures of mononuclear cells from APL patients. In one case, cells derived from a patient refractory to RA failed to respond to the drug in vitro. RA resistance of the cells was reversed by combination RA/HMBA treatment. In two other cases, RA-mediated differentiation was augmented threefold by sequential RA/HMBA treatment. A clinical strategy that combines intermittent low doses of RA and nonretinoid agents may increase clinical response and at the same time help prevent RA resistance in APL patients. HMBA and phenylacetate are currently undergoing clini-
cal trials and have nonoverlapping toxicities with RA. Therefore, sequential combination differentiation therapy may be suitable for clinical evaluation in newly diagnosed patients with APL or in cases refractory to current treatment programs.

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