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Advances in Brief

Inhibitors of Histone Deacetylase Relieve ETO-mediated Repression and Induce Differentiation of AML1-ETO Leukemia Cells

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Abstract

The (8;21) translocation, found in 12% of acute myeloid leukemia (AML), creates the chimeric fusion product, AML1-ETO. Previously, we demonstrated that the ETO moiety recruits a transcription repression complex that includes the histone deacetylase (HDAC1) enzyme. Here, we used inhibitors of HDAC1 to study the pathophysiology of AML1-ETO. Both the potent inhibitor, trichostatin (TSA), and the well-known but less specific inhibitor, phenylbutyrate (PB), could partially reverse ETO-mediated transcriptional repression. PB was also able to induce partial differentiation of the AML1-ETO cell line, Kasumi-1. With the intention of developing a clinically useful protocol, we combined PB with a number of other agents that induced differentiation and apoptosis of Kasumi-1 cells. In summary, transcriptional repression mediated by AML1-ETO appears to play a mechanistic role in the t(8;21) AML, and relief of repression using agents such as PB (alone or in combination) may prove to be therapeutically useful.

Introduction

Approximately 30% of French-American-British (FAB) class M2 AML[3] cases have a translocation between chromosomes 8 and 21, creating the chimeric fusion product AML1-ETO (1–4). AML1 is a transcription factor that regulates the expression of a number of genes involved in hematopoiesis, including some of the homeobox-containing *HOX* genes known to play an important role in development (5, 6). The AML1-ETO fusion blocks *trans*-activation of AML1-dependent target genes (7–10), thereby disrupting the expression of myeloperoxidase, neutrophil elastase, interleukin 3, and GM-CSF, among others. Recently, the ETO moiety in the AML1-ETO fusion has been shown to interact with the nuclear receptor corepressor (N-CoR) complex (11). This results in recruitment of Sin3 and the histone deacetylase (HDAC1) enzyme to AML1-dependent promoters. Histone deacetylation is thought to play an important role in transcriptional repression, and its involvement in disrupting AML1 function may be critical to leukemogenesis in the t(8;21) AML.

The model that we and others have proposed whereby a histone deacetylase is recruited to the local environment by AML1-ETO to repress transcription of genes that would otherwise be activated by AML1 is analogous to the story that has been unfolding for APL (12, 13). In APL, the t(15;17)-derived PML-RAR fusion protein interacts with the N-CoR/Sin3/HDAC1 repression complex in a ligand-dependent manner; however, the N-CoR complex is released only in the presence of pharmacological doses of RA. Thus, in the presence of physiological levels of RA, PML-RAR continues to repress transcription from RA-dependent genes. In the t(11;17) variant of APL, this situation is exacerbated; the PLZF-RAR fusion binds N-CoR at a second, ligand-independent site and thus becomes a constitutive repressor of the retinoid-responsive genes. Inhibitors of HDAC1 can reverse N-CoR-mediated repression, and synergistically with RA, reverse the APL phenotype in APL cells (14). On the basis of the success of HDAC1 inhibitors in APL, we asked whether an inhibitor of HDAC1 would alter the leukemic phenotype induced by AML1-ETO. We also tested a number of other compounds that have been reported to induce differentiation and/or apoptosis in the t(8;21) AML cell line, Kasumi-1 (15), and other AML cell lines. Both G-CSF (16) and dexamethasone (17) have been reported to induce apoptosis of Kasumi-1 cells, whereas arsenic trioxide (18) can induce cell death in a number of myeloid leukemia lines and has been reported recently to induce remission in relapsed APL patients (19).

Materials and Methods

Transcription Repression Assays. The ETO coding region was fused to the GAL4 DBD in the pFA-CMV plasmid (Stratagene) to create the GAL4-DBD/ETO expression plasmid (11). The luciferase reporter gene was driven by the thymidine kinase promoter with four copies of the GAL4 DNA-binding sequence upstream (provided by B. O'Malley, Baylor Medical School, Houston, TX). NIH/3T3 cells were transfected with the reporter plasmid and 80 ng of either GAL4-DBD/ETO or the GAL4-DBD plasmid (pFA-CMV) by calcium phosphate coprecipitation. After transfection, TSA (ICN Biomedicals, Inc.) or PB (Triple Crown America, Inc., Perkasie, PA) was added to the culture medium, and the cultures were maintained for 16–36 h and then lysed. Luciferase and β-galactosidase (as internal control of transfection efficiency) assays were performed as described previously (11).

Differentiation and Apoptosis Induction. The Kasumi-1 cell line (15) was cultured in 24-well plates at a concentration of 2×10^5 cells/ml in RPMI media supplemented with 15% fetal bovine serum and treated with PB (in PBS), dexamethasone (in ethanol), arsenic trioxide (in PBS), and G-CSF and GM-CSF (both in PBS and 0.5% BSA) at the stated concentrations. In dexamethasone-treated cell cultures, the final concentration of alcohol was 0.1%. Cells were harvested at day 7 of culture for flow cytometric analysis, WST-1 tetrazolium dye viability assays, trypan blue exclusion assays, and cytospin preparation.

Flow Cytometric Analysis. Cells were subjected to centrifugation and resuspended in 100 μl of 1× binding buffer (HEPES buffered saline solution with 2.5 mM CaCl₂; R & D Systems, Minneapolis, MN). PE-labeled annexin V (PharMingen, San Diego, CA) and fluorescein (FITC)-labeled CD13 (Immunotech, Marseilles, France) were added, and the cells were incubated for 30 min in the dark at room temperature. After the addition of 400 μl of 1× binding buffer and agitation, flow cytometric analysis was performed using a Coulter EPICS XL-MCL. Results are reported as the percentage of positive cells compared with an isotype control.

Viability Assays. Ten μl of WST-1 dye stock (Boehringer Mannheim, Indianapolis, IN) was added to 100 μl of cell suspension (or media alone as control) in a 96-well plate. Cells were incubated for 2 h at 37°C, 5% CO₂ prior to dual wavelength analysis (450 and 600 nm) on a Bio-Rad 3550 Microplate Reader. Results were reported in absorbance units.

Cytospin Preparation. Five hundred μl of cell suspension were deposited onto glass slides in a Shandon Cytospin 2 centrifuge. Cells were then stained with Wright-Giemsa stain.

Results

TSA and PB, Inhibitors of HDAC1, Partially Reverse ETO-mediated Transcription Repression. In transient transfection assays using a luciferase reporter gene, ETO potently represses transcription (47-fold repression, as shown in Fig. 1). However, the repression effects of ETO were dramatically reduced by the addition of TSA at concentrations of 100 and 200 nM (19- and 17.5-fold, respectively; Fig. 1*A*). PB, a well-known but less specific inhibitor of HDAC1, could also partially relieve ETO-mediated repression at PB concentrations of 1 mM or higher, although dose dependence was not observed (Fig. 1*B*). These results suggested that inhibition of HDAC1 activity could partially reverse ETO-mediated repression.

Differentiation and Apoptosis of Kasumi-1 Cells with PB. CD13 is a pan-myeloid marker, present on mature granulocytes and monocytes. The proportion of cells expressing the CD13 antigen increased from 20% to >60% after 7 days of culture with 1–3 mM PB (Fig. 2). Addition of G-CSF had an additive effect on CD13 induction (Fig. 2). Cytospin preparations of treated cells showed morphological changes of partial monocytic differentiation and apoptosis, particularly after treatment with PB and G-CSF (Fig. 3).

In Fig. 4, we have tabulated CD13 expression (*panel A*) and annexin staining (*panel B*) in Kasumi-1 cells treated with PB, G-CSF, GM-CSF, dexamethasone, and arsenic trioxide alone or in combination with each other. G-CSF, GM-CSF, and arsenic trioxide alone produced <10% increases in CD13 expression (compared with untreated controls; Fig. 4*A*). Dexamethasone and arsenic trioxide were not as potent as PB in induction of CD13 expression, but they induced greater apoptosis and decreased cell viability.

Apoptosis and cell viability were assessed using annexin-PE staining (Fig. 4*B*), forward scatter/side scatter profiles by flow cytometry, tetrazolium salt cleavage (WST-1), and trypan blue exclusion. PB produced a <10% increase in annexin-staining cells over controls (Fig. 4*B*), with trypan blue exclusion assays showing cell viability
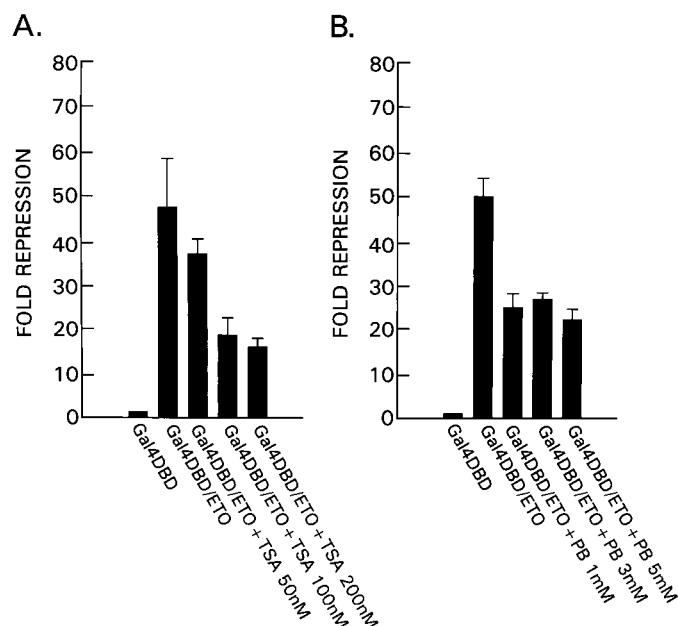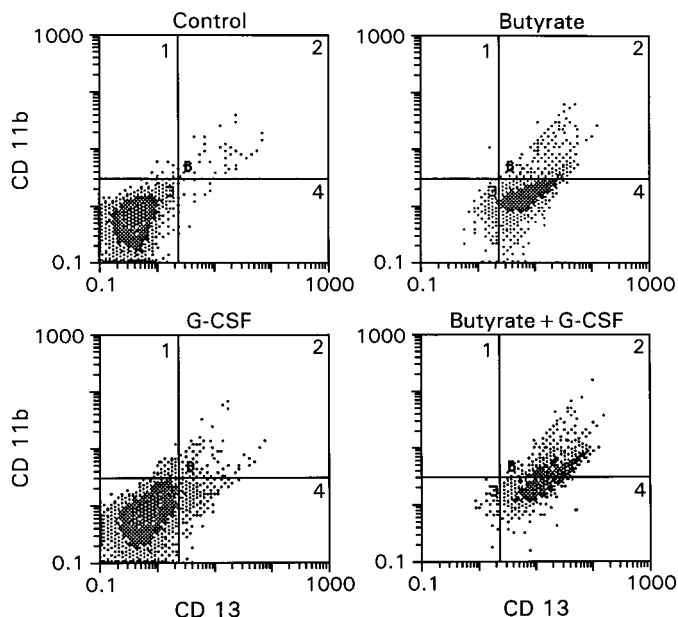

Fig. 2. Scatterplots of CD13 and CD11b expression in Kasumi-1 cells 7 days after incubation with 3 mM PB alone, 100ng/ml of G-CSF, and a combination of PB and G-CSF.

about half that of control samples. (The lower cell numbers may have been a result of growth-inhibitory properties of PB.) Approximately 60% of annexin-positive cells were also CD13 positive in both PB and control samples (data not shown).

G-CSF and GM-CSF produced slight increases in annexin staining and decreased the number of viable cells as assessed by WST-1 viability assays. Combinations of the above agents had additive effects on induction of apoptosis in Kasumi-1 cells. As assessed by WST-1 assays (data not shown), PB alone produced a 30% decrease in cell viability, dexamethasone produced a 90% decrease, arsenic trioxide produced an 80% decrease, and the combination of agents produced a 98% decrease in cell viability.

Kasumi-1 cells are known to express the CD34 antigen. Dual staining with CD13-PE and CD34-FITC was performed to assess whether increased CD13 expression was accompanied by decreased CD34 expression (data not shown). Three patterns of CD13 and CD34 expression were induced by the various agents tested. PB alone induced CD34-bright/CD13-bright cells, whereas G-CSF and GM-CSF induced CD34-dim cells without a marked increase in CD13 expression. The potent inducers of apoptosis, dexamethasone and arsenic, induced CD34-dim/CD13-bright cells. Decreased CD34 and increased CD13 expression correlated with apoptosis induction; live cells (as indicated by forward scatter/side scatter) maintained a CD34-bright phenotype without increased CD13 expression.

Discussion

Transcriptional repression may underlie the pathophysiology of APL, t(8;21) AML, and other myeloid leukemias. In these disorders, the nuclear receptor corepressor (N-CoR) complex appears to play an important role in aberrantly mediating transcriptional repression. Reversal of repression mediated by the t(8;21) AML and APL fusion proteins may be a useful therapeutic strategy, as illustrated by the success of HDAC1 inhibitors in APL (14). We show here that in transient transfection assays, the HDAC1 inhibitors, TSA or PB, partially reversed transcriptional repression mediated by the ETO oncoprotein. As yet, however, no experiments have formally demonstrated "rescue" of expression of the hematopoietic target genes (such

Fig. 1. ETO has potent transcription repression effects that could be overcome partially by the addition of TSA (*A*) or PB (*B*). Fold repression was calculated as described in "Materials and Methods." *Bars,* SD.
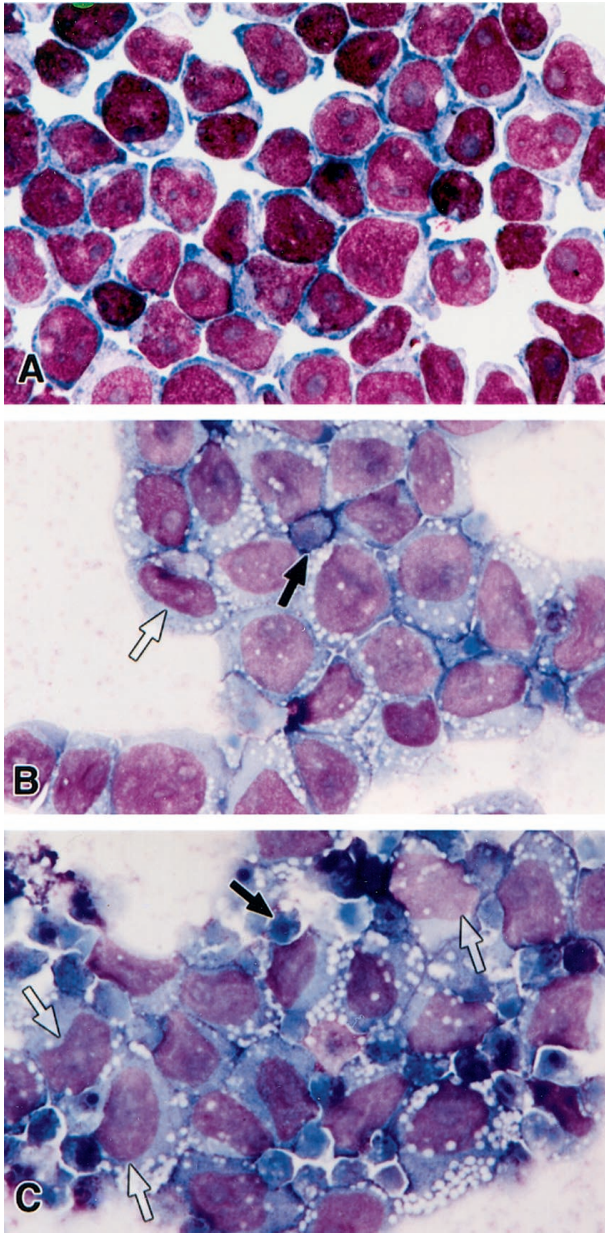
Fig. 3. Cytospin preparations of Kasumi-1 cells were Wright-Giemsa stained 7 days after incubation with 3 mM PB and 100ng/ml of G-CSF. Morphological changes of monocytic differentiation (*white arrows*) and apoptosis, with nuclear condensation and vacuolization (*black arrows*), were seen. *A*, control, untreated; *B*, 3 mM PB; *C*, 3 mM PB + 100ng/ml of G-CSF.

ase, a hallmark of differentiated macrophages (20). Our experiments used inhibitors of histone deacetylase as a means of potentially reversing AML1-ETO-mediated gene repression and promoting differentiation. Treatment of the AML1-ETO cell line Kasumi-1 with PB increased expression of the pan-myeloid marker CD13 and induced morphological changes consistent with monocytic differentiation. This differentiation was only partial, even at higher concentrations of PB. G-CSF, GM-CSF, and arsenic trioxide also increased CD13 expression but not to the same extent as PB. Rather, arsenic trioxide and dexamethasone were effective inducers of apoptosis.

Additive effects on differentiation were seen with the combination of PB and G- or GM-CSF (see Figs. 2 and 4). This may be because the AML1-ETO fusion associates with (and inhibits the function of) CCAAT enhancer binding protein-α (21), which normally heterodimerizes with AML1 and activates myeloid cell-specific promoters, including those for cytokine receptors (GM-CSF, G-CSF, and macrophage-CSF receptors) and granule protein (neutrophil elastase). Treatment with PB would be predicted to rescue wild-type AML1 and CCAAT enhancer binding protein-α function, promoting granulocytic differentiation and cytokine-responsive signal transduction pathways.

In some model systems, differentiation signaling itself can induce apoptosis, and such a mechanism could partly explain apoptosis induction by PB, G-CSF, and GM-CSF in Kasumi-1 cells. However, PB-induced CD13 expression was not accompanied by a decrease in CD34 expression. Whereas the most potent inducers of apoptosis, dexamethasone and arsenic, induced little or no increase in overall CD13 expression, treatment resulted in the appearance of distinct populations of cells with decreased CD34 expression and increased
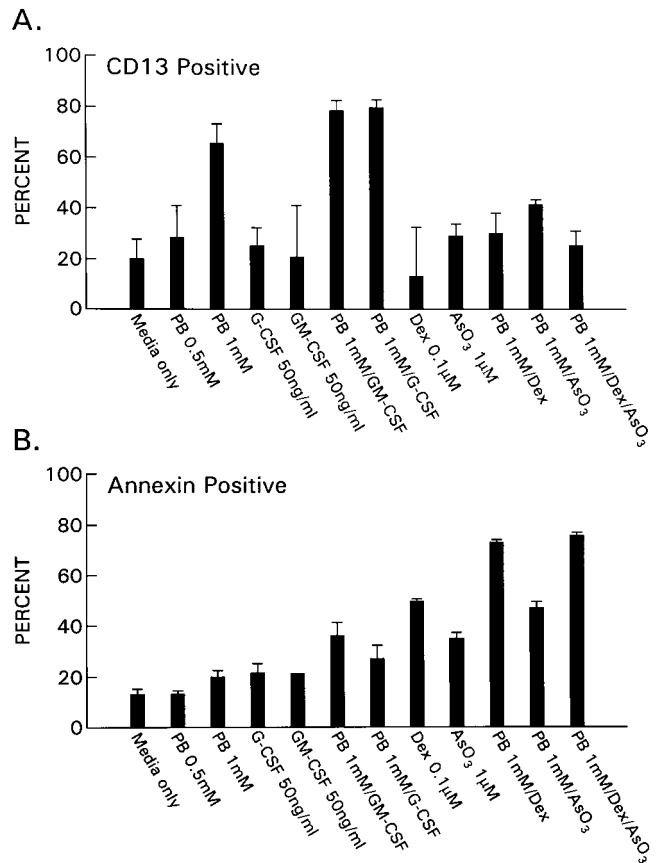

Fig. 4. Kasumi-1 cells were analyzed using flow cytometry for CD13-FITC as a differentiation marker (*A*) and annexin-PE as an apoptosis marker (*B*), 7 days after incubation with the various agents shown. Experiments were repeated two to four times; *bars,* SD. Dex, dexamethasone; AsO₃, arsenic trioxide.

as myeloperoxidase, interleukin 3, and others), disrupted by AML1-ETO, in a relevant cell culture model. With this caveat in mind regarding the molecular explanation of our results, we also showed that treatment of Kasumi-1 cells with PB (at 1 mM concentration, same as in the transcription assays) induced partial differentiation and apoptosis of the leukemic cells. With the intention to identify a combination that would be valuable clinically, a number of other agents that have been reported to produce differentiation and apoptosis of leukemia cell lines were also tested against Kasumi-1 cells. A combination of PB (at clinically attainable levels), dexamethasone, and arsenic trioxide was particularly effective in inducing apoptosis.

A specific link between AML1-ETO expression and inhibition of differentiation has been suggested by a number of experiments (5, 20); in particular, Kasumi-1 cells treated with an AML1-ETO-specific antisense oligonucleotide were induced to express nonspecific ester-

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CD13 expression, which were dying cells. Despite these suggestions that differentiation signaling played some role in apoptosis induction with all of the agents tested, only 40–60% of the annexin-positive cells were also CD13 positive, and other nondifferentiation-related pathways were likely to have been operational. Apoptosis induction by PB is, therefore, not a result of terminal differentiation and probably not directly analogous to RA-induced apoptosis of APL cells.

Combinations of PB with G-CSF or GM-CSF had an additive or synergistic effect on induction of CD13 expression, whereas combinations with dexamethasone or arsenic had additive effects on induction of apoptosis. The different patterns of differentiation and apoptosis produced by the different agents suggest different pathways of activity. If such combinations of agents prove to be therapeutic *in vivo*, perhaps in combination with chemotherapy, they would offer the advantages of low toxicity, low cost, and possibly increased efficacy.

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