

Decitabine and Vitamin D3 differentially affect hematopoietic transcription factors to induce monocytic differentiation

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Abstract. Standard chemotherapy is not curative for many patients with acute myeloid leukemia (AML). New treatment strategies combining demethylating agents, such as decitabine, and drugs that induce myelomonocytic differentiation (i.e. Vitamin D3) may re-establish functional hematopoiesis in these patients. We studied the effects of decitabine alone or in combination with Vitamin D3 (VD3) on U937 cells and AML blasts. Preincubation with decitabine (0.1-1 μ M) and subsequent exposure to VD3 (3 nM) synergistically induced monocytic differentiation. To elucidate the mechanisms of decitabine- and VD3-induced monocytic differentiation, we investigated the effects of the two drugs on transcription factors implicated in monocytic differentiation. Northern and Western blotting showed that decitabine induced transcription of c-jun but not PU.1, while VD3 increased PU.1, IRF8, and C/EBPbeta but not c-jun. Using electromobility shift assays, we demonstrated increased DNA binding of nuclear proteins from decitabine- and VD3-induced U937 cells to the CD11b promoter. In addition, we investigated whether the myeloid transcription factor Sp1 played a role in decitabine- and VD3-induced CD14 expression. Indeed, we found that mithramycin A, a specific inhibitor of Sp1, inhibited both VD3- and decitabine-induced upregulation of CD14, which is in line with previous data showing that Sp1 is critical for CD14 promoter activity. Induction of CD11b and/or CD14 by decitabine and/or VD3 was confirmed in primary AML patient samples at the time of diagnosis. In conclusion,

decitabine synergizes with Vitamin D3 to induce CD11b and CD14 expression, likely by enhancing PU.1/c-jun and Sp1 transcriptional activity.

Introduction

Human malignancies are increasingly seen as a bulk of differentiated tumor cells lacking self-renewal potential and a smaller fraction of tumor stem cells which possess the capacity to both self-renew and show signs of differentiation into their progeny. These malignant progenitors usually have an increased proliferative potential and decreased susceptibility to undergo apoptosis. In the case of acute myeloid leukemias, the bulk tumor mass consists of malignant myeloid blasts which have lost the potential to differentiate into mature effector cells. The resulting replacement of mature cells of the erythrocytic, myeloid, lymphoid, and megakaryocytic lineages by leukemic blasts puts patients with AML at an increased risk for infections, hemorrhages, and organ failures, the major causes of death in this patient population.

While conventional antileukemic strategies using chemotherapy can eradicate the blast cell population and induce a complete remission in about 70% of patients with AML, few patients achieve long-term cure (1). This is most likely due to the persistence of the malignant stem cell population that has not been eradicated by the chemotherapy. Additional strategies are therefore needed to eliminate the leukemic stem cells (2).

One approach is the specific induction of differentiation of the leukemic cells into functional effector cells such as mature granulocytes. This approach is highly successful in acute promyelocytic leukemia (APL) where the addition of ATRA results in significantly improved long-term cure rates (3). However, other subtypes of AML do not respond to ATRA treatment to this extent highlighting the need for additional differentiation-inducing agents. This requires the knowledge of the specific mechanisms that are responsible for the differentiation block. Preclinical studies including mutant mouse models have shown that transcription factors play a decisive role in normal hematopoietic differentiation (4).

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Interestingly, mutations in transcription factor genes (i.e. CEBPA, Runx1, PU.1) occur in a subset of AML cases. However, far more commonly, transcription factor deregulation is caused by decreased expression of these factors, and this is mainly due to methylation and/or histone deacetylation of gene regulatory elements such as promoters and enhancers. Substances that target these transcriptional alterations are currently intensely studied and include demethylating agents and histone deacetylase inhibitors (HDACI) (5).

The present study was performed to define the impact of the demethylating agent, decitabine, alone or in combination with the differentiation inducer, Vitamin D3 (VD3), on the differentiation of U937 cells and blasts from patients with AML, and to study potential mechanisms involved in differentiation induction.

Materials and methods

Cells and reagents. U937 cells were purchased from ATCC and maintained in RPMI supplemented with 10% fetal bovine serum (FBS) (Hyclone, UT, USA), 1% penicillin/streptomycin (P/S), and 1% glutamine (both from Sigma, Munich, Germany). Mononuclear cells from the bone marrow of patients with AML were Ficoll-isolated and frozen in liquid nitrogen until use. After thawing, they were cultured in IMDM (Invitrogen, Karlsruhe, Germany) supplemented with 20% FBS, 1% P/S, and 1% glutamine. Informed consent was obtained from all patients. Decitabine was used at 0.1 or 1 μ M, Vitamin D3 at 3 or 100 nM, and Mithramycin A at concentrations ranging from 10 to 100 nM as indicated (all purchased from Sigma). Cell number and viability were assessed using a Neubauer chamber after trypan blue staining. Morphologic differentiation of U937 cells was assessed after 8 days of culture by Wright-Giemsa staining.

Flow cytometry. CD11b, CD14, and CD15 expression was assessed at the indicated times by flow cytometry using anti-CD11b, anti-CD14, and anti-CD15 antibodies and a FACS-Calibur flow cytometer (all from Becton Dickinson, Heidelberg, Germany) (6).

Western blot analysis. Cell lysates were prepared as described (6). Antibodies against the following proteins were used: PU.1 antibody, *c-jun*, Vitamin D receptor, interferon regulatory factor 8 (IRF8), CCAAT enhancer binding protein alpha and beta (CEBPA and CEBPB), Sp1, and interleukin-18 (IL-18) (all antibodies purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Northern blot analysis. Total RNA was isolated from stimulated cells after 2 days of stimulation using the RNEasy Mini kit (Qiagen, Hilden, Germany), 20 μ g of RNA was separated on a 1% agarose gel containing formaldehyde (0.22 M) and transferred to a Biotrans Plus membrane (MP Biomedicals, Irvine, CA, USA) by capillary transfer overnight in 10X SSC. Membranes were crosslinked by ultraviolet light exposure (Stratagene, La Jolla, CA, USA) and subsequently probed with the [³²P- α]-dCTP radiolabelled probes for human *c-jun* [1.3 kb HindIII-BglII fragment of *c-jun* cDNA in pS3H (7)] or VDR [2 kb EcoRI fragment of VDR cDNA in pSG5 (8)].

Electromobility shift assay (EMSA). Nuclear extracts were essentially prepared as described (9). Briefly, nuclear extracts were isolated from 5x10⁷ U937 cells (9), and protein was assayed using the Bio-Rad kit (Bio-Rad, Munich, Germany). CD11b promoter oligonucleotides (10) were annealed and labeled at the 5'-ends using [³²P]-ATP (Amersham, Arlington Heights, IL, USA) and T4 polynucleotide kinase (NEB, Beverly, MA, USA) and separated from unincorporated nucleotide by passage through a Sephadex G-25 column (Boehringer-Mannheim, Mannheim, Germany). EMSAs were performed by incubating 10 μ g of nuclear extracts with 50,000 counts per minute (cpm) double-stranded oligonucleotide in a 20- μ l reaction mixture containing 10 mM HEPES-KOH buffer (pH 7.9), 50 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, 10% glycerol, 1 μ g acetylated bovine serum albumin (NEB, Ipswich, MA, USA), and 0.5 μ g poly(dI-dC) at 25°C for 20 min. To assess the presence of PU.1 in the binding complex, 1 μ l polyclonal anti-*c-jun* antibody (Santa Cruz Biotechnology) was added to the binding reaction. Binding reactions were resolved on a 4% non-denaturing polyacrylamide gel containing 1X TBE (0.089 M Tris borate, 0.089 M boric acid, and 0.002 M EDTA) and electrophoresed at 150 V at 4°C. Oligonucleotides used in EMSA were derived from the human CD11b promoter (bp -26 to +2, with the PU.1 binding site underlined): 5'-GCTCAAAGAAGGGCAG AAAAGGAGAAGTAGG (10).

Statistical analysis. Data are expressed as mean \pm standard deviation (SD) and were compared using a Student's t-test.

Results

Decitabine synergizes with Vitamin D3 but not with TGF β to induce monocytic differentiation of U937 cells. Induction of monocytic differentiation by a combination of Vitamin D3 (VD3) and transforming growth factor beta-1 (TGF β) is well established for U937 cells (11). We analyzed the effects of the demethylating agent, decitabine, on U937 cell differentiation.

Since a previous study had found optimal synergism between decitabine and another inducer of differentiation, all-*trans* retinoic acid (ATRA), when cells were pre-incubated with decitabine and then stimulated with ATRA (12), we primed U937 cells with decitabine one day prior to adding VD3. Using this approach, 0.1-1 μ M of decitabine alone induced CD11b and CD14 surface expression 3- to 18-fold and 3- to 4-fold, respectively, within 3 days (Fig. 1A and B). VD3 (3 nM) induced CD11b and CD14 expression 5- to 10-fold and 1.5- to 8-fold, respectively. Decitabine synergized with VD3 to increase CD11b and CD14 surface marker expression up to 73-fold and 11-fold, respectively (Fig. 1A and B). There was no change in viability by exposure to either drug (Fig. 1C). Vitamin D3 induced a reproducible increase of cell number, and this was inhibited by increasing doses of decitabine (Fig. 1D). Decitabine alone did not consistently alter the number of cells in culture.

Morphologic analysis of U937 cells after an 8-day exposure to even low doses of decitabine (0.1 μ M) showed strong evidence of monocytic differentiation, including a decrease of the nucleus-to-cytoplasm ratio, a decrease of basophilic cytoplasmic appearance, an increase of overall cell size, and

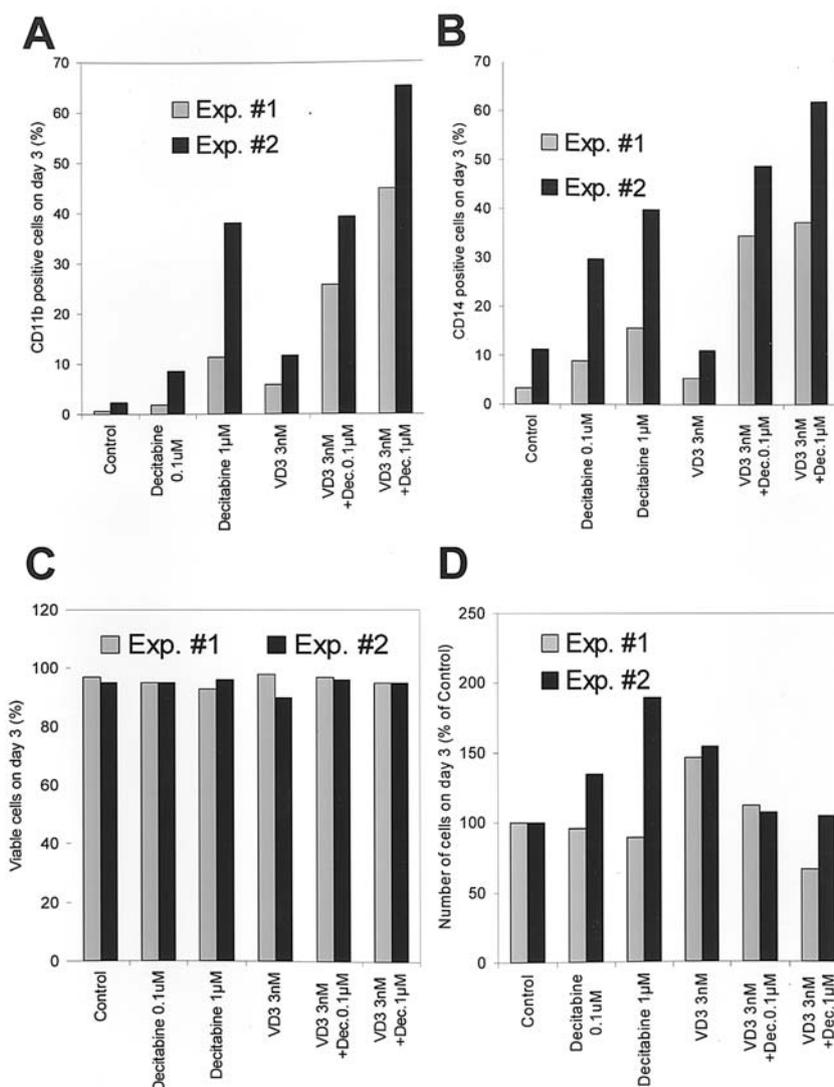


Figure 1. Effects of decitabine and Vitamin D3 on differentiation, cell number, and viability. (A and B) U937 cells were cultured in the presence or absence of decitabine (1 μ M, added on day 0), Vitamin D3 (3 nM, added on day 1), or both, as indicated in the figure. The percentage of CD11b and CD14 positive cells was assessed by flow cytometry after 3 days of culture. Data from two independent experiments (Exp. nos. 1 and 2) are shown. (C and D) U937 cells were cultured as detailed in A and assessed for cell number and viability by trypan blue exclusion. Data from two independent experiments are shown (Exp. nos. 1 and 2).

the induction of cytoplasmic vesicles (Fig. 2A). At higher doses of decitabine (1 μ M), monocytic differentiation was still evident, but there was a concomitant increase of cell death within these cultures after 8 days. Low doses of Vitamin D3 (3 nM) induced moderate differentiation towards monocytic cells. Interestingly, the toxicity of high doses of decitabine were inhibited by combined treatment with Vitamin D3, and there was maximal monocytic differentiation after 8 days with a combination of decitabine (1 μ M) and VD3 (3 nM) (Fig. 2A).

Decitabine and Vitamin D3 induce monocytic differentiation in primary AML blasts. Ficoll-enriched leukemic blasts from 17 patients with AML were exposed to 1 μ M decitabine in the presence or absence of 100 nM Vitamin D3 for 7 days. Cells in cultures containing decitabine alone showed significantly decreased viability in 6 of 17 cases [patient nos. 3, 7 (both $p < 0.05$ vs. control), 13 ($p = 0.056$), 16 ($p < 0.05$), 18 ($p = 0.067$)

and 20 ($p < 0.05$, Fig. 3A), as detected by trypan blue exclusion. Five of these patients (all except patient no. 3) also showed signs of differentiation by flow cytometry, detected by significant upregulation of either CD11b (Pt. no. 16), CD15 (Pt. no. 20) (Fig. 2B), or CD14 (Pt. nos. 7, 13 and 18) (all $p < 0.05$ vs. control) (Fig. 3B). Altogether, CD11b, CD14, or CD15 were induced by decitabine in five (29%), four (24%), and four (24%) of all patients, respectively, by Vitamin D3 in nine (53%), ten (59%), and eleven (65%), respectively, and by both agents together in ten (59%), ten (59%), and nine (53%) of all patients, respectively (Fig. 3 and data not shown).

Decitabine but not Vitamin D3 induces c-jun expression. The effects of decitabine and VD3 on the expression of important hematopoietic transcription factors were investigated. Decitabine (1 μ M) but not VD3 (3 nM) induced the expression of c-jun mRNA and protein within 3 days of culture (Fig. 4A). At the same time, VD3 enhanced the

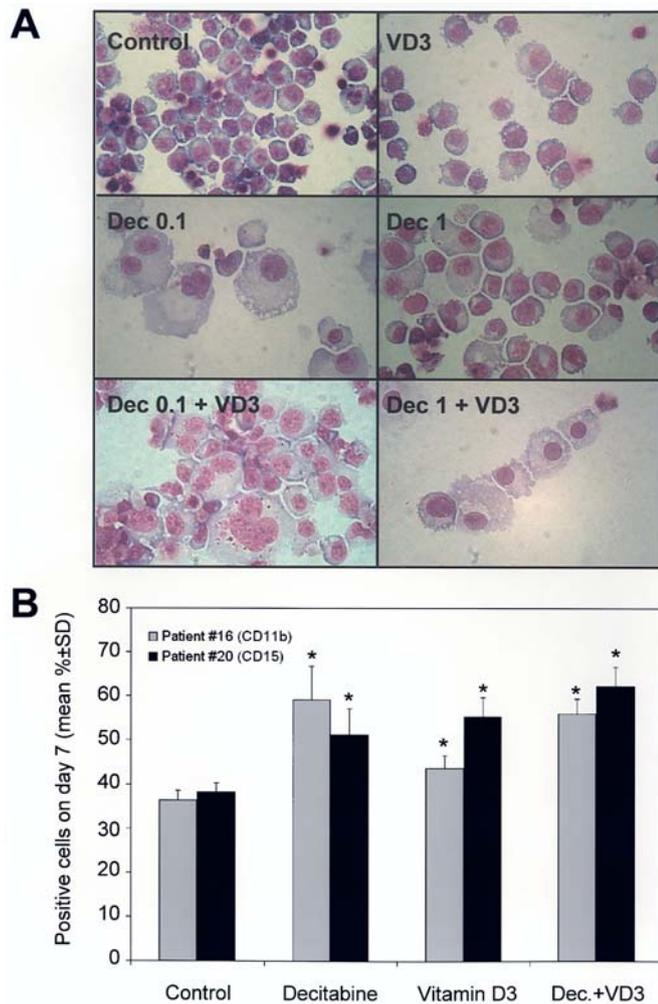


Figure 2. Induction of monocytic differentiation by decitabine and/or VD3 in U937 cells and primary AML blasts. (A) U937 cells were cultured in the presence or absence of decitabine (Dec. 0.1 or 1 μ M), Vitamin D3 (VD3 3 nM), or both, as indicated, and cell morphology was assessed on day 8 of culture using Wright-Giemsa staining. (B) Ficoll-enriched leukemic blasts isolated from the bone marrow of patients nos. 16 and 20 with acute myeloid leukemia were cultured in the presence or absence of decitabine (1 μ M), Vitamin D3 (10⁻⁷ M), or both, for 7 days, and the surface marker expression of CD11b (patient no. 16) or CD15 (patient no. 20), respectively, was analyzed flow cytometrically. The percentages of positive cells are shown as mean \pm SD from triplicate cultures. * p <0.05 vs. control.

expression of PU.1, interferon-regulatory factor-8 (IRF8), and CCAAT enhancer binding protein beta (CEBPB) proteins (Fig. 4B and C). In the presence of VD3, increased levels of PU.1 were detectable both in the nucleus and cytoplasm of the cells (Fig. 4B and C). No significant effects were seen on PU.1 mRNA or Sp1, Vitamin D receptor, interleukin-18, or CEBPA protein expression regardless of the presence of decitabine or VD3 (Fig. 4B and C).

Decitabine enhances transcription factor binding to the CD11b promoter. In electromobility shift assays, decitabine and Vitamin D3 both separately and together enhanced the formation of DNA binding complexes (solid arrow) to a human CD11b promoter probe (Fig. 5A). The intensity of this complex strongly decreased but was not completely

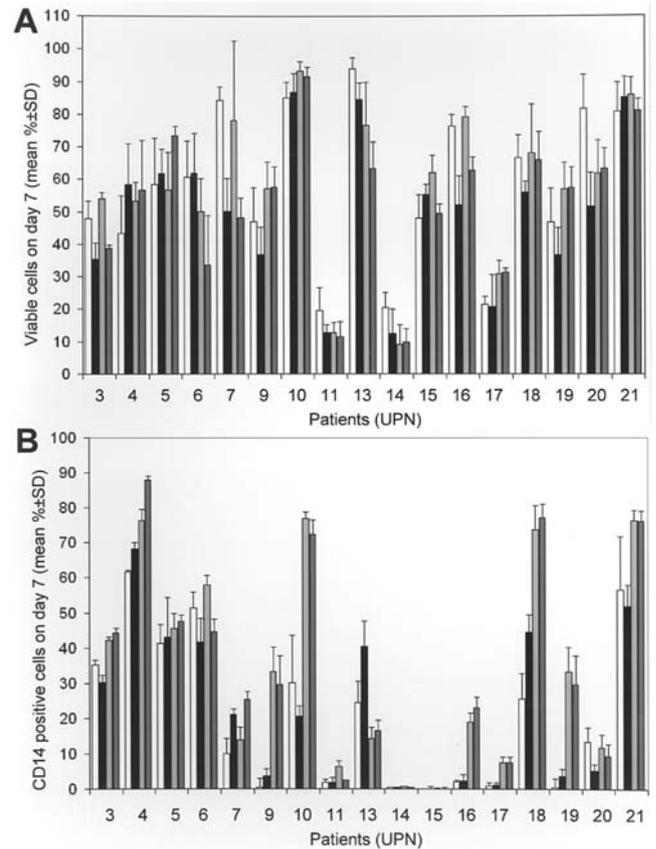


Figure 3. Effect of decitabine and/or Vitamin D3 on the viability and differentiation of AML blasts. (A) Ficoll-separated leukemic blasts from the bone marrow of 17 patients with AML were subjected to a 7-day culture in the absence (white bars) or presence of decitabine (1 μ M; black), Vitamin D3 (10⁻⁷ M; light grey), or both (dark grey), and analyzed for viability by trypan blue exclusion. UPN designates unique patient number. All experiments were performed in triplicate (except for patient no. 14, where there were only enough cells for one replicate). (B) Assessment of CD14 surface marker expression by flow cytometry in cells from the same 17 patients cultured as described above. For significant differences, see Results.

abrogated after the addition of anti-PU.1 antibody suggesting the presence of PU.1 in this protein-DNA complex but also the presence of additional binding partners (Fig. 5A).

Mithramycin, an inhibitor of Sp1 function, inhibits CD14 but not CD11b expression in decitabine- and VD3-induced U937 cells. Binding of Sp1 to the CD14 promoter is critical for CD14 promoter activity during monocytic differentiation (13). Since Sp1 protein expression was unaltered by decitabine and/or VD3 (Fig. 4), we investigated the possibility of altered Sp1 function in the presence of decitabine and VD3. Indeed, 75 nM of mithramycin, an inhibitor of Sp1 transcription factor binding, significantly inhibited Vitamin D3-induced CD14 (p <0.05) but not CD11b expression (p =0.15) (Fig. 5B). There was a dose-dependent decrease of CD14 expression upon mithramycin treatment (Fig. 5B). Mithramycin also inhibited CD14 expression in the presence of both decitabine and VD3, while CD11b expression remained unaltered (Fig. 5B). Addition of mithramycin (75 nM) to VD3-containing cultures significantly decreased the number of cells by 48% (p <0.05) but did not change cell viability (p =0.37) (data not shown).

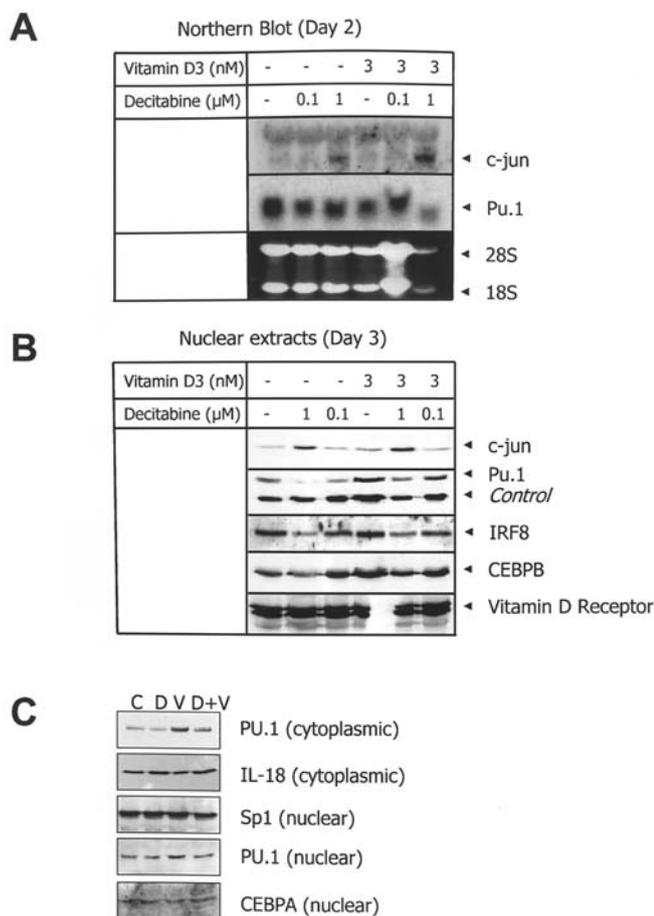


Figure 4. Transcription factor changes in the presence of decitabine and Vitamin D3. (A) U937 cells were cultured in the presence or absence of decitabine (0.1 or 1 μ M, added on day 0) and/or Vitamin D3 (3 nM, added on day 1), total RNA was extracted, and Northern blotting was performed on day 2 of culture. Expression of c-jun and PU.1 was detected using specific probes as described in Materials and methods. As a loading control, 18S and 28S RNA is shown. (B) Nuclear extracts were prepared from U937 cells cultured as described above and subjected to Western blotting. The expression levels of c-jun, PU.1, IRF8, CCAAT enhancer binding protein beta (CEBPB), and Vitamin D receptor were analyzed using specific antibodies against these proteins. (C) U937 cells were cultured as above, and protein expression of cytoplasmic PU.1 and interleukin-18 and nuclear PU.1, Sp1, and CEBPA was assessed by Western blotting. C, control; D, decitabine (1 μ M, added on day 0), V, Vitamin D3 (100 nM, added on day 0), D+V, decitabine and Vitamin D3.

Discussion

The discovery of the impact of epigenetic regulation on gene expression has stimulated a variety of novel treatment approaches to human malignancies. Demethylating agents and histone deacetylase inhibitors are currently under intense preclinical and clinical investigation for the treatment of human leukemias (14). Promising results have already been obtained with decitabine in patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) where this agent has led to significant responses including complete remissions (15-17). Intriguingly, even patients that are known to fare poorly with conventional chemotherapy, such as patients with an unfavorable karyotype, showed remarkable responses to decitabine treatment (17). In addition, low doses of decitabine were at least as effective as high doses (17), the time to treatment response was longer than

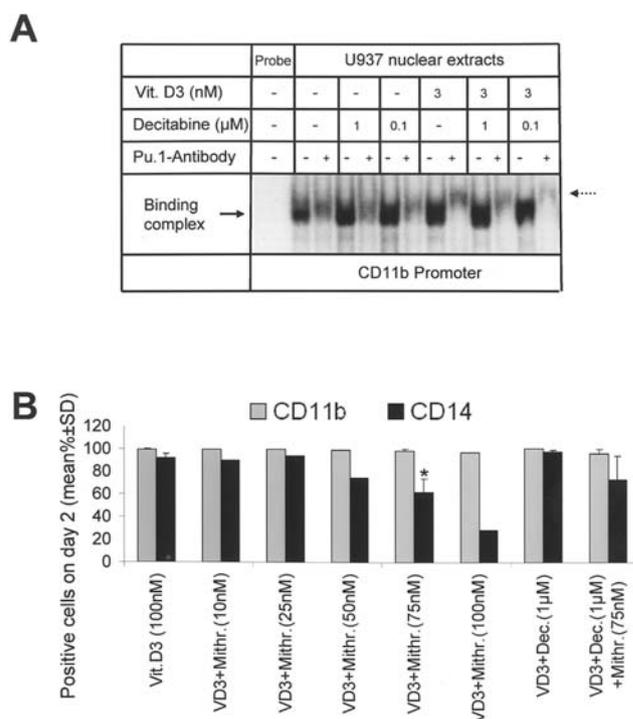


Figure 5. DNA complexes binding to the CD11b promoter in the presence of decitabine and/or Vitamin D3. (A) Nuclear cell extracts from decitabine- or Vitamin D3- (added after 1 day) stimulated U937 cells were prepared after 3 days of culture and analyzed for DNA binding activity to oligonucleotides representing the human CD11b promoter (bases -26 to +2). The solid arrows point to the DNA binding complex. The change of mobility of the binding complexes remaining after addition of the anti-PU.1 antibody is indicated by a dashed arrow. 'Probe' indicates that no nuclear extracts were added in this lane. (B) U937 cells were cultured in the presence of Vitamin D3 (100 nM, added on day 0) alone or in combination with decitabine (1 μ M, added on day 0), and exposed to mithramycin (10-100 nM, added on day 0). The percentage of CD11b and CD14 positive cells on day 2 is shown. * $p < 0.05$ vs. Vitamin D3 alone.

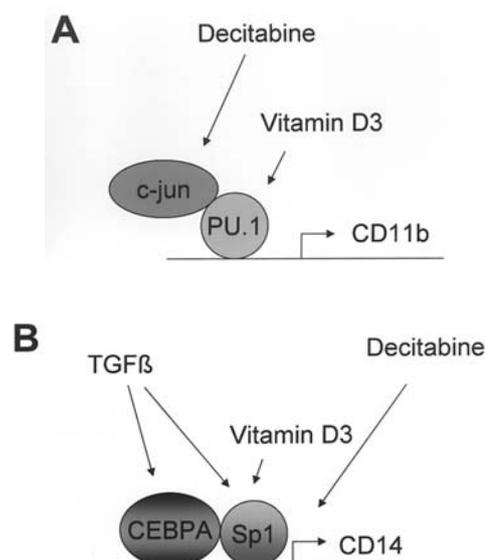


Figure 6. Diagram of differential transcription factor requirements for the activation of the human CD11b and CD14 promoters. The potential stimulatory effects of decitabine and Vitamin D3 as well as of transforming growth factor beta (TGF β) on the expression and/or function of the hematopoietic transcription factors c-jun, PU.1, CCAAT enhancer binding protein alpha (CEBPA), and Sp1 are indicated.

with conventional chemotherapy (15,17), and genome-wide demethylation progressed even after the elimination of leukemic blasts from the peripheral blood and bone marrow (18).

These findings suggest that decitabine not only induces apoptosis of leukemic cells but that it durably changes chromatin structure and function. Differentiation induced by demethylating agents might be relevant for the beneficial effects. Here, we confirm and extend previous data (12) showing that decitabine induces monocytic differentiation of U937 cells, potentiates Vitamin D3 induced differentiation, and inhibits VD3-induced cell proliferation without compromising cell viability (Figs. 1 and 2A). Decitabine also significantly increased CD11b, CD14 and/or CD15 expression in blast cells from patients with AML (Figs. 2B and 3). Preexisting CD11b, CD14, or CD15 expression levels were not predictive for the response to decitabine (data not shown). To investigate the molecular basis of decitabine-induced differentiation and its enhancement of VD3-induced differentiation, we analyzed the expression of hematopoietic transcription factors and their function in the stimulated cells. C-jun expression by TGF β , which has long been used to enhance VD3-induced differentiation of U937 cells (11), was found to be enhanced by VD3 in osteoblastic MC3T3-E1 cells, and this required functional Vitamin D receptor (19). In the present study, decitabine but not VD3 induced c-jun mRNA and protein expression in U937 cells (Fig. 4). Intriguingly, a recent study investigating genome-wide methylation using ChIP-on-chip technology discovered the c-jun promoter to be frequently methylated on CpG islands in U937 cells (20). C-jun expression was low in parental cells but increased with decitabine treatment (20). Interestingly, c-jun induction was not seen with VD3 treatment in our experiments, suggesting that c-jun upregulation was specific for decitabine.

Given that c-jun and PU.1 are both critical for monocytic differentiation and frequently bind to each other in DNA-binding complexes, we investigated whether PU.1 was induced by VD3 or decitabine. We found that PU.1 protein was increased by VD3 (Fig. 4) and that both decitabine and VD3 separately and in combination enhanced DNA binding of U937 nuclear extracts to the human CD11b promoter as indicated by electromobility shift assays (EMSAs) (Fig. 5A). These EMSAs also showed that PU.1 was present in this complex but that the complex was not completely abrogated by the addition of PU.1 antibody (Fig. 5A), suggesting the presence of additional binding partners (i.e. c-jun). Taken together, these data show that the combination of decitabine and VD3 enhanced both c-jun and PU.1 expression, and this may provide an explanation for the synergistic activation of the CD11b promoter by both substances. PU.1 binding to the CD11b promoter has been firmly established using both DNA footprinting and gel shift assays (10). While PU.1 is able to bind to DNA [CD11b or MCSFR promoter (21)], c-jun itself cannot bind to these promoters (21). Thus, by titrating the amount of active c-jun, decitabine may be able to synergize with other drugs to induce monocytic differentiation. c-jun has been described to be one of the most significantly down-regulated proteins in the differentiation of granulocyte-macrophage progenitor cells (GMP) into mature monocytes in preleukemic mice that lack a critical upstream regulatory

element of the PU.1 gene, and re-expression of c-jun in these preleukemic cells partially restored differentiation (Steidl, *et al*, ASH Annual Meeting Abstracts 106: 463, 2005), again emphasizing the need for both factors in hematopoietic cell differentiation.

According to the present study, the mechanisms of CD14 and CD11b gene activation differ in their requirement for specific transcription factors. CD14 expression was induced by high doses of Vitamin D3 (100 nM, Fig. 5B), but even lower doses of VD3 (3 nM) were able to synergize with decitabine and enhance decitabine-induced CD14 expression (Fig. 1B). We found that CD14 but not CD11b expression was significantly reduced by the Sp1 transcription factor inhibitor, mithramycin (Fig. 5B), suggesting that Sp1 plays a critical role in the activation of the CD14 promoter but is less critical for the CD11b promoter. This is well in line with reports that have demonstrated binding and transactivation of the CD14 promoter by Sp1 (13,22). Additionally, the binding of CEBPA was described to be critical for TGF β -induced CD14 promoter activation with TGF β increasing the expression of CEBPA (23). In our experiments, we found no alteration of CEBPA expression by decitabine, suggesting that decitabine may synergize with VD3 to induce CD14 by enhancing Sp1 function.

Recent advances using demethylating agents alone or in combination with HDACI in patients with AML or MDS syndromes have been made (15,16,18,24). Vitamin D3 itself has been shown to mediate ligand-dependent acetylation of its target gene PPAR δ by recruiting the histone acetyltransferase CBP (25). Many tumor suppressor or differentiation-inducing genes are heavily methylated in blasts from patients with AML (20,26-28) and other cancers (29-32). Our data suggest differential and possibly independent mechanisms of decitabine- and VD3-induced promoter activation (Fig. 6) and thus provide further rationale for clinical trials investigating the synergistic action of both demethylating and differentiation-inducing agents and/or HDAC inhibitors in patients with AML, MDS, and solid tumors.

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