Decitabine Maintains Hematopoietic Precursor Self-Renewal by Preventing Repression of Stem Cell Genes by a Differentiation-Inducing Stimulus

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Abstract

The cytosine analogue decitabine alters hematopoietic differentiation. For example, decitabine treatment increases self-renewal of normal hematopoietic stem cells. The mechanisms underlying decitabine-induced shifts in differentiation are poorly understood, but likely relate to the ability of decitabine to deplete the chromatin-modifying enzyme DNA methyltransferase 1 (DNMT1), which plays a central role in transcription repression. HOXB4 is a transcription factor that promotes hematopoietic stem cell self-renewal. In hematopoietic precursors induced to differentiate by the lineage-specifying transcription factor Pu.1 or by the cytokine granulocyte-colony stimulating factor, there is rapid repression of HOXB4 and other stem cell genes. Depletion of DNMT1 using shRNA or decitabine prevents HOXB4 repression by Pu.1 or granulocyte-colony stimulating factor and maintains hematopoietic precursor self-renewal. In contrast, depletion of DNMT1 by decitabine 6 hours after the differentiation stimulus, that is, after repression of HOXB4 has occurred, augments differentiation. Therefore, DNMT1 is required for the early repression of stem cell genes, which occurs in response to a differentiation stimulus, providing a mechanistic explanation for the observation that decitabine can maintain or increase hematopoietic stem cell self-renewal in the presence of a differentiation stimulus. Using decitabine to deplete DNMT1 after this early repression phase does not impair progressive differentiation.

Introduction

The cytosine analogue 5-aza-2′-deoxycytidine (decitabine), approved by the U.S. Food and Drug Administration as a treatment for myelodysplastic syndrome, alters hematopoietic differentiation. Therapeutically important differentiation-altering effects of decitabine include terminal differentiation of leukemia cells (1–5), increased erythropoiesis and fetal hemoglobin to treat sickle cell disease and β-thalassemia (6, 7), and increased self-renewal of hematopoietic stem cells, which could have a role in ex vivo expansion of hematopoietic stem cells (8–10). The mechanisms underlying these decitabine-induced shifts in differentiation are poorly understood, but likely relate to the ability of decitabine to deplete the chromatin-modifying enzyme DNA methyltransferase 1 (DNMT1; ref. 11). DNMT1 duplicates methylation marks onto the newly synthesized DNA strand during mitosis (maintenance methylation; ref. 12). DNMT1 is also a component of histone methyltransferase protein complexes that create methylation marks of transcription repression (reviewed in ref. 13) and histone demethylase protein complexes that remove methylation marks of transcription activation (14). Accordingly, decitabine treatment to deplete DNMT1 not only hypomethylates DNA but can also extensively modify chromatin, producing decondensation of chromatin structure (15).

Drugs that relax chromatin by inhibiting histone deacetylase (HDAC) produce changes in hematopoietic differentiation similar to that produced by decitabine: terminal differentiation of leukemia cells (4, 16, 17), increased fetal hemoglobin expression (reviewed in ref. 18), and increased normal hematopoietic stem cell self-renewal (8, 19, 20). Because a shared property of HDAC inhibitors and DNMT1 depletion by decitabine is antagonism of transcription repression, this suggests that antagonism of transcription repression could have a mechanistic role in producing the observed shifts in differentiation by decitabine and by HDAC inhibitors.
The transition from one differentiation level to the next likely involves repression of key genes that maintain the initial or starting differentiation level. Here, we examined the possibility that decitabine antagonizes the repression of stem cell–associated genes by a differentiation-inducing stimulus. We used two in vitro models of hematopoiesis to examine this possibility: a model of lineage-specifying transcription factor (Pu.1)–driven differentiation of murine hematopoietic cells and a model of cytokine [granulocyte-colony stimulating factor (G-CSF)]–driven differentiation of human hematopoietic cells. The generated observations provide a mechanistic explanation for some of the differentiation-altering effects of decitabine treatment and can guide the application of this drug.

Materials and Methods

**Generation of PUER cells with stable suppression of Dnmt1 expression**

A lentiviral vector, pLenti6-DEST (Invitrogen), was used to construct short hairpin (sh) RNA for Dnmt1. The specific 21-bp target sequences for mouse shDnmt1 (5′-GAACGGCATCAAGTGGAAC-3′) were synthesized in sense and antisense orientations by Advanced DNA Technology; the single-strand oligos were then annealed to form double-strand oligos and subsequently ligated with pENTRY vector (Invitrogen) downstream of an RNA promoter. The ligated constructs were transformed into *E. coli* TOPO10. Positive clones were verified by DNA sequencing. The verified clones were then recombinated into pLenti6-DEST vector using the Invitrogen ViralPack kit, resulting in pLenti6-Dnmt1. The pLenti6-Dnmt1 constructs were then transfected together with envelope-encoding plasmid (VSVG) into 293FT packaging cell line to produce lentivirus. The supernatant containing lentivirus was harvested at 48 hours after transfection. Titers were determined on NIH3T3 cells as transducing units using serial dilutions of vector stocks with 8 μg/mL polybrene (Sigma Chemical).

PUER cells (21) are murine hematopoietic precursor cells that have been retrovirally transduced to express Pu.1 fused to the estrogen receptor. To knock down Dnmt1 in cells, murine PUER cells were grown in phenol red–free Iscove’s modified Eagle’s medium with 10% fetal bovine serum, 2.5 ng/mL mouse interleukin-3 (IL-3), 1 μg/mL puromycin, 55 μmol/L β-mercaptoethanol, 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO2 in air. The lentivirus-containing supernatant was added to the cell culture at appropriate concentration with 8 μg/mL polybrene. Twenty-four hours after infection, 4 μg/mL blasticidin was added to the cell culture for positive clone selection. The blasticidin-resistant cells were analyzed for Dnmt1 expression by real-time quantitative PCR (RQ-PCR) and Western blot. Control cells were PUER transduced with empty vector (PUER control).

Addition of 4-hydroxytamoxifen (OHT) to PUER triggers their terminal differentiation into macrophages (21). Differentiation status was analyzed by (a) presence of adherent cells by light microscopy, (b) morphologic changes in Giemsa stained cytopsin preparations, and (c) c-Kit (eBioscience) and F4/80 (eBioscience) expression by flow cytometry. Cell analysis was done on a Coulter Epics XL-MCL flow cytometer equipped with CXP software (Beckman-Coulter).

**Cell fractionation and protein extraction**

Approximately 5 million PUER control and PUER shDnmt1 cells were used to prepare cell lysates. After removal of the medium, cells were transferred to 15-mL conical tubes and washed twice with 5 mL of ice-cold 1× PBS. Cells were resuspended in 500 μL of 1× hypotonic buffer containing 10 mmol/L HEPES, 1.5 mmol/L MgCl2, 10 mmol/L KCl, 0.5 mmol/L DTT, 10 mmol/L phenylmethylsulfonfluryl fluoride, and protease inhibitor cocktail (Sigma-Aldrich, A8340) and incubated for 10 minutes on ice. Twenty microliters of 10% NP40 were added to cell suspensions to break the cell membrane. After another 10-minute incubation on ice, cell suspensions were centrifuged at 500 × g for 10 minutes. The supernatant was transferred to clean 1.5-mL Eppendorf tubes and labeled as the cytoplasm fraction. Nuclear pellets were washed twice with ice-cold water and resuspended in 50 μL of 50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L MgCl2, 10 mmol/L phenylmethylsulfonfluryl fluoride, protease inhibitor cocktail (Sigma-Aldrich, A8340), and DNase 1 (Sigma-Aldrich, D5915, final concentration of 50 units). The nuclear suspensions were incubated on ice for 30 minutes with vigorous vortexing every 5 minutes. At the end of incubation, 50 μL of protein extraction buffer containing 4% SDS, 10 mmol/L DTT, 20% glycerol in 50 mmol/L Tris-HCl were added and the mixture was sonicated on ice and centrifuged at 140,000 rpm for 5 minutes. The supernatant containing nuclear proteins was transferred to clean tubes and protein concentration was determined by bicinchoninic acid assay.

**One-dimensional SDS-PAGE and Western blotting**

Approximately 50 μg of cytoplasmic and nuclear protein extracts from PUER control and PUER shDnmt1 cells, together with molecular weight markers, were subjected to one-dimensional SDS-PAGE on 4% to 12% gradient gels (Invitrogen). After electrophoresis per manufacturer’s manual (Invitrogen), proteins were transferred onto polyvinylidene difluoride membranes (Millipore) at 35-V constant voltage for 1 hour using an Invitrogen semidry blotting apparatus. Western analyses of polyvinylidene difluoride membranes used established protocols and rabbit monoclonal anti-Pu.1 (Cell Signaling, #2258) and anti-β-actin peroxidase (Sigma- Aldrich, A3854).

**Real-time PCR**

mRNA levels were assayed using RQ-PCR. Briefly, total cellular RNA was isolated from 5 × 10⁶ cells using RNeasy
Plus (Qiagen) according to the manufacturer’s protocol. For cDNA synthesis, after a denaturation step of 5 minutes at 70°C, 1 µg of RNA was reverse transcribed to single-stranded cDNA using a mix of random hexamers and oligo-dT primers and Moloney murine leukemia virus reverse transcriptase for first-strand synthesis (Promega). Real-time PCR was done with Real-time PCR Master Mix containing SYBR Green I and hotstart Taq DNA polymerase (Takara). GAPDH was amplified as control. Primer sequences are available on request. Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNA will be detected using the iCycler instrument (Bio-Rad). Data are reported as relative expression values, which was determined by raising 2 to the power of the negative value of ΔΔCT for each sample.

Treatment of cells with decitabine

Decitabine stock solution (5 mmol/L) was generated by reconstituting lyophilized decitabine in 100% methanol. Stock solution was stored at −20°C for up to 3 weeks. Working solution was generated by diluting the stock solution 1:100 in PBS immediately before addition to the cells at a further dilution as per the intended final concentration. Similar amounts of methanol are added to untreated control cells. Cells were treated with decitabine (0.5 µmol/L) with timings as designated per the text and figure legends.

Normal cord blood samples

Umbilical cord blood was collected during normal full-term deliveries following written informed consent of the mother on a Case Institutional Review Board–approved protocol.

Isolation of CD34+ cells

CD34+ cells from umbilical cord blood were immunopositively purified using a magnetic cell sorting system, CD34 MicroBead Kit (Miltenyi Biotec), according to the manufacturer’s instructions. The purity of the CD34+ population (ranged typically from 95% to 99%) was determined by immunolabeling with FITC-conjugated monoclonal antibodies against CD34 (clone 581, Beckman Coulter) that reacted with an epitope other than the antibody used for separation.

Human hematopoietic cell culture and clonogenic progenitor assays

CD34+ selected normal human hematopoietic cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum and 10 ng/mL of the following human cytokines: stem cell factor (SCF), FLT3 ligand (FLT3), thrombopoietin (TPO), IL-3, and IL-6. Cells were treated once with 0.5 µmol/L decitabine either concurrently with the addition of 50 ng/mL G-CSF or 6 hours after 50 ng/mL G-CSF. Cells were harvested from liquid culture on day 9 and plated into methylcellulose at a concentration of 20,000 cells/mL of semisolid medium (Methocult, Stem Cell Technologies) supplemented with SCF (50 ng/mL), granulocyte macrophage colony-stimulating factor (10 ng/mL), IL-3 (10 ng/mL), and erythropoietin (3 units/mL). Colonies were counted and photographed on day 12 of semisolid culture.

Results

Dnmt1 depletion by shRNA prevents Pu.1-induced HoxB4 repression and differentiation

Hematopoietic cell fate is driven by key transcription factors such as HoxB4, which promotes hematopoietic stem cell self-renewal (22), and the lineage-specifying factor PU.1, which is required for macrophage and B-cell lineage commitment and differentiation (reviewed in ref. 23). We wished to examine if Dnmt1 is required for the repression of HoxB4 and other stem cell–associated genes by Pu.1. PUER cells are murine Pu.1−/− hematopoietic precursors that have been transduced with a retroviral vector to express Pu.1 fused to the estrogen receptor (21). In cell culture with murine IL-3 (mIL-3), PUER cells self-renew indefinitely. Addition of OHT to these cells (as an estrogen agonist) causes functional reintroduction of Pu.1 by translocation into the nucleus and triggers terminal macrophage differentiation (21).

Although not normal hematopoietic stem cells, PUER, self-renewing in mIL3, express high levels of the self-renewal promoting factor HoxB4 and other genes that are expressed at high levels in stem cells, Bmi-1 and c-Kit. After addition of OHT, there is repression of HoxB4, Bmi-1, and c-Kit, followed by activation of genes expressed at high levels in macrophages [macrophage colony stimulating factor receptor (Mcsfr), granulocyte monocyte colony stimulating factor (Gmcsfr), and F4/80; Fig. 1A]. These expression changes were accompanied by acquisition of morphologic changes of macrophage differentiation (increase in cell size, decrease in nuclear–cytoplasmic ratio, clumping of nuclear chromatin, adherence to culture plates, and cytoplasmic vacuolization; Fig. 1A).

Dnmt1 was knocked down in PUER by lentiviral transduction of shRNA (PUER shDnmt1). Control cells were PUER transduced with empty vector (PUER control). Knockdown of Dnmt1 by shDnmt1 was >70% by Western blot (Fig. 1B).

Addition of OHT to shDnmt1 cells caused Pu.1-ER to be rapidly translocated from the cytoplasm into the nucleus at high levels (Fig. 1C).

OHT-treated control cells rapidly repressed expression of stem cell genes HoxB4, Bmi-1, and c-Kit; however, OHT-treated shDnmt1 cells continued to express high levels of these stem cell genes (Fig. 1D). OHT-treated control cells activated expression of differentiation genes Mcsfr, Gmcsfr, and F4/80; however, these genes remained relatively repressed in OHT-treated shDnmt1 cells (Fig. 1D).

OHT-treated control cells displayed extensive morphologic changes of macrophage differentiation (Fig. 1D). In contrast, OHT-treated shDnmt1 cells continued to display morphologic immaturity, with most cells resembling untreated shDnmt1 cells (Fig. 1D).
OHT treatment substantially decreased the proliferation of control cells. OHT treatment also decreased the proliferation of shDnmt1 cells, but not to the same extent as the decrease in proliferation of control cells (OHT-treated shDnmt1 cells continued to expand exponentially; Fig. 1E). Therefore, depletion of Dnmt1 prevented Pu.1-mediated repression of HoxB4 and other stem cell genes, prevented differentiation, and allowed hematopoietic precursor self-renewal in the presence of nuclear Pu.1.

Maintenance of hematopoietic precursor self-renewal by decitabine requires timing of treatment to prevent Pu.1-mediated stem cell gene repression

To further develop this observation, decitabine, the cytosine analogue that binds and depletes Dnmt1, was added to the PUER cells concurrent with the introduction of Pu.1 (concurrent with OHT) or 6 hours after introduction of Pu.1 (6 hours after OHT), that is, after the repression of HoxB4 had occurred.

OHT treatment of PUER caused rapid repression of the stem cell genes HoxB4, Bmi-1, and c-Kit, coupled with activation of the differentiation genes F4/80, Gmcsfr, and Mcsfr. Decitabine addition concurrent with OHT attenuated the repression of the stem cell genes and the activation of the differentiation genes. Decitabine addition 6 hours after OHT did not significantly impede stem cell gene repression or the subsequent differentiation gene activation (Fig. 2A).

OHT treatment of PUER caused rapid macrophage differentiation. Decitabine concurrent with OHT largely prevented morphologic differentiation, with most cells resembling cells without OHT. Decitabine 6 hours...
after OHT resulted in more extensive changes of macrophage differentiation than with OHT alone (Fig. 2A).

OHT treatment of PUER caused a substantial decrease in proliferation compared with untreated cells. Decitabine concurrent with OHT also decreased cell proliferation compared with untreated cells, but not to the same extent as OHT alone. Decitabine 6 hours after OHT terminated cell proliferation (Fig. 2B).

OHT treatment of PUER caused a decrease in c-Kit protein expression and an increase in F4/80 protein expression as measured by flow cytometry. Decitabine concurrent with OHT significantly attenuated the decrease in c-Kit expression and the increase in F4/80 expression produced by OHT alone. Decitabine 6 hours after OHT resulted in c-Kit and F4/80 expression changes that resembled OHT alone (Fig. 2B).

Therefore, the ability of decitabine to maintain self-renewal of hematopoietic precursors in the presence of nuclear Pu.1 depends on timing of decitabine treatment in relationship to the Pu.1 differentiation stimulus.

**Maintenance of human hematopoietic precursor self-renewal in the presence of G-CSF requires timing of decitabine treatment to prevent stem cell–associated gene repression by G-CSF**

Previously, we and others showed that treating human CD34+ hematopoietic stem and progenitor cells with decitabine maintains or promotes their self-renewal in vitro such that decitabine can be used for ex vivo expansion of hematopoietic stem and progenitor cells (8, 9, 24).

Human CD34+ cells express high levels of HOXB4 and other factors known to be associated with stem cell self-renewal such as the stem cell factor receptor (c-KIT) and the polycomb protein BMI-1 (Fig. 3A). Addition of the myeloid differentiation–promoting cytokine G-CSF to these cells results in rapid repression...
of the stem cell genes *HOXB4*, *BMI-1*, and *c-KIT*, coupled with activation of the differentiation genes *PU.1*, *MCSFR*, and *G-CSF* receptor (*G-CSFR*; Fig. 3A). Addition of decitabine concurrent with *G-CSF* significantly attenuated repression of the stem cell genes and attenuated activation of the differentiation genes compared with *G-CSF* alone. Addition of decitabine 6 hours after *G-CSF* did not impede stem cell gene repression or differentiation gene activation compared with *G-CSF* alone (Fig. 3A).

*G-CSF* induced morphologic differentiation of the cells (increased cell size, decreased nuclear-cytoplasmic ratio, chromatin clumping, and increased granulation; Fig. 3A). Decitabine addition concurrent with *G-CSF* largely prevented the morphologic differentiation seen with *G-CSF* alone (Fig. 3A). In contrast, decitabine addition 6 hours after *G-CSF* produced more extensive morphologic changes of differentiation compared with cells treated with *G-CSF* alone (Fig. 3A).

Decitabine addition concurrent with *G-CSF* decreased the number of progenitors present on day 9 (9 days after addition of *G-CSF*) of liquid culture (progenitors were assayed by plating cells harvested from liquid culture into semisolid media) compared with *G-CSF* alone. However, the colonies formed were larger, consistent with more immature character of the progenitors (Fig. 3B). In contrast, decitabine addition 6 hours after *G-CSF* (after *HOXB4* repression and *PU.1* activation) produced a greater decrease in colony number (decreased progenitors) and colony size (increased maturity of progenitors) compared with cells treated with concurrent decitabine and *G-CSF* or *G-CSF* alone (Fig. 3B).

Decitabine addition concurrent with *G-CSF* decreased cell proliferation compared with *G-CSF* alone, but not to the same extent as decitabine 6 hours after *G-CSF* (Fig. 3C).

Therefore, the effects of decitabine treatment on human hematopoietic cell fate depend on timing of treatment in relationship to the cytokine differentiation stimulus.

**Discussion**

The repression of *HOXB4* and other stem cell genes that occurs early after a differentiation stimulus is necessary for differentiation to proceed. This suggests a mechanistic basis for the observation that drugs that antagonize transcription repression can increase hematopoietic stem cell self-renewal (8–10, 19, 20, 24–26). DNMT1 depletion by decitabine, before or concurrent with a differentiation-inducing stimulus, by preventing the repression of stem
cell genes by the differentiation stimulus, maintains stem cell self-renewal. Consistent with the proposed mechanism, treatment with decitabine shortly after a differentiation stimulus (after transition through the early repression phase) does not maintain self-renewal and instead augments differentiation.

An alternative model to explain the ability of decitabine to increase hematopoietic stem cell self-renewal is that decitabine treatment to deplete DNMT1 directly reactivates a stem cell program in the treated cells. The different cell fate produced by treating cells with decitabine before versus after a differentiation stimulus is more consistent with the proposed model than this alternative. Emphasizing that the cell fate consequences of decitabine treatment are cell context dependent, decitabine treatment induces terminal differentiation of leukemia cells (1–5), including the demonstrably self-renewing subset of leukemia-initiating cells, in contrast to its effect on normal hematopoietic stem cells.

Another question is whether the differentiation-altering effects of decitabine can be attributed to DNMT1 depletion rather than to other effects of the drug. Decitabine is a cytosine analogue, and therefore, as per the class effect of nucleoside analogues, it can induce DNA damage and cytotoxicity. However, decitabine is rapidly cleaved and degraded by hydrolysis (27). Hence, decitabine is substantially less efficient at impeding DNA replication machinery and terminating DNA strand elongation than an equimolar concentration of cytosine arabinoside (ara-C), the cytosine analogue that is the mainstay of cytotoxic acute myelogenous leukemia therapy (28, 29). We have shown that the concentrations of decitabine used here deplete DNMT1 without causing measurable DNA damage or apoptosis in normal human hematopoietic precursors. Others have shown that decitabine depletes the maintenance DNA methyltransferase DNMT1 (11), but not the de novo DNA methyltransferases DNMT3A and DNMT3B (11), consistent with the S-phase specificity of decitabine. The similar shifts in differentiation produced by HDAC inhibitors and decitabine are also consistent with a mechanism that relates to antagonism of transcription repression. Finally, conditional deletion of DNMT1 by genetic methods shows that it has a role in regulating hematopoietic cell fate. In a murine model of inducible knockout of Dnmt1 in adult mice, defects in the hematopoietic stem cell compartment and in progenitors suggested that Dnmt1 is required for hematopoietic stem cell self-renewal and differentiation (30). In another genetic study, Dnmt1 was partially depleted using a hypomorphic Dnmt1 allele (31). An increase in hematopoietic stem cells and deficiencies in lineage-committed progenitors were noted (31).

Although the murine conditional Dnmt1 knockout models show a role for Dnmt1 in hematopoietic differentiation, these genetic models differ from DNMT1 depletion by decitabine treatment in important respects: DNMT1 depletion by decitabine is transient, with recovery of DNMT1 protein in normal hematopoietic precursors to close to pretreatment levels within 96 hours of decitabine exposure; the transient DNMT1 depletion by decitabine, although substantial, is not complete (3, 11); DNMT1 depletion by decitabine is S-phase dependent and is therefore more extensive in actively cycling cells such as hematopoietic precursors than in supporting tissue such as stroma.

The findings here suggest that DNMT1, a key chromatin modifying enzyme involved in transcription repression, is required for the early repression of stem cell genes that occurs in response to a differentiation stimulus. This repression step is necessary for differentiation to proceed to the next level. The varied cell fate consequences of decitabine treatment reflect the differentiation phase– and cell context–dependent role of the DNMT1 molecular target of therapy.

Disclosure of Potential Conflicts of Interest

Y. Saunthararajah: previous consultant for HemaQuest, a company developing treatments for blood diseases such as thalassemia and sickle cell disease. No other potential conflicts of interest were disclosed.

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