Histone Deacetylase Inhibitors (HDI) Cause DNA Damage in Leukemia Cells: A Mechanism for Leukemia-Specific HDI-Dependent Apoptosis?

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Abstract

Histone deacetylase inhibitors (HDI) increase gene expression through induction of histone acetylation. However, it remains unclear whether increases in specific gene expression events determine the apoptotic response following HDI administration. Herein, we show that a variety of HDI trigger in hematopoietic cells not only widespread histone acetylation and DNA damage responses but also actual DNA damage, which is significantly increased in leukemic cells compared with normal cells. Thus, increase in H2AX and ataxia telangiectasia mutated (ATM) phosphorylation, early markers of DNA damage, occurs rapidly following HDI administration. Activation of the DNA damage and repair response following HDI treatment is further emphasized by localizing DNA repair proteins to regions of DNA damage. These events are followed by subsequent apoptosis of neoplastic cells but not normal cells. Our data indicate that induction of apoptosis by HDI may result predominantly through accumulation of excessive DNA damage in leukemia cells, leading to activation of apoptosis. (Mol Cancer Res 2006;4(8):563–73)

Introduction

Histone deacetylase inhibitors (HDI) abrogate the action of histone deacetylases, a group of enzymes that control global chromatin architecture through histone modification. In turn, inhibition of histone deacetylation results in chromatin relaxation and increased access of transcriptional elements to promoters, leading to enhanced gene activity (1-3). Leukemias are seen as particularly good candidates for HDI therapy. These agents result in apoptosis of cancer cells in vivo and are now undergoing phase I/II clinical trials (4). In these diseases, specific oncogenic fusion proteins can recruit histone deacetylases to the promoters of their target genes and these events may constitute major steps in leukemogenesis (5).

Recently, it has been suggested that chromatin changes induced by HDI can directly activate the DNA damage pathway (6). Activation of ataxia telangiectasia mutated (ATM) kinase seems to be an initiating event in cellular responses to irradiation. ATM activation is not dependent on direct binding to DNA strand breaks but may result from changes in the structure of chromatin (6).

It has been widely reported that HDI induces p21waf and/or Fas ligand binding that could ultimately result in apoptosis of tumor cells (7). Several HDI, including valproic acid and suberoylanilide hydroxamic acid, show antileukemic activities correlating with overexpression of the cell cycle inhibitor p21waf (8, 9). Recently, it has been reported that, in addition to p21waf, HDI induce tumor necrosis factor-related apoptosis-inducing ligand (Apo2L, TNFSF10) by directly activating the TNFSF10 promoter, thereby triggering tumor-selective death signaling in acute myeloid leukemia (AML) cell lines, murine acute promyeloctic leukemia (APL) cells in vivo, and cells from patients with AML (10, 11).

Herein, we have investigated the alternative possibility that HDI might induce actual DNA damage that then triggers apoptotic events. We find that, within minutes of HDI treatment, DNA damage is detected in the form of regions of ssDNA that is coincident with acetylation of histone H4. Importantly, apoptosis, as detected by caspase-3 and poly(ADP-ribose) polymerase cleavage, is only detected some hours following HDI treatment in vitro and in vivo. Furthermore, abrogating the expression of key molecules in the DNA damage and repair response, such as H2AX and ATM, leads to an accumulation of DNA damage and accelerated apoptosis in HDI-treated cells. Importantly, leukemic cells that overexpress BCL2 still show DNA damage, while apoptosis is abolished. Thus, although apoptosis may be triggered by HDI, this is likely in some instances to occur downstream of DNA-damaging events.
Results

HDI have been shown to differentially induce apoptosis in cancer and leukemic cells (10, 11). However, the mechanism underlying the action of HDI is still not entirely understood. We examined the possibility that HDI might actually induce DNA damage and then trigger apoptotic pathways. We first determined the relationship of acetylated histone H4 and phosphorylation of H2AX in normal and leukemic cells following HDI treatment. We first found that addition of HDI to normal and leukemic cells results in histone acetylation and rapid induction of variant histone H2AX phosphorylation on Ser139. This DNA damage event signals for DNA repair and rapid induction of variant histone H2AX phosphorylation following HDI treatment (Fig. 2B and C) following HDI treatment but still manifesting apoptosis, because K562 cells stably transfected with antiapoptotic BCL2 (Fig. 2A) inhibit caspase cleavage (Fig. 2B and C) following HDI treatment but still induce DNA damage as measured by γH2AX (Fig. 2D). We also note that BCL2 transfectants treated with HDI show more DNA damage foci at an earlier time point following HDI treatment than K562 controls (Table 1). In addition, overall histone acetylation is not associated per se with apoptosis induced by other non-HDI agents. Thus, addition of the proapoptotic agent staurosporine (1 μmol/L) also resulted in H2AX phosphorylation at 240 minutes but with no concomitant increase in histone H4 acetylation in HL60 (Fig. 1G) and normal PBL (Fig. 1H).

All of this data suggest that, specifically in leukemic cells, therapeutic doses of HDI cause DNA damage in association with the acetylation of histone proteins and that apoptosis subsequently follows these events. To confirm that HDI caused DNA damage in leukemic cells, we searched directly for evidence of DNA damage on chromatin fibers using an established assay for DNA damage that relies on the fact that an anti–bromodeoxyuridine (BrdUrd) antibody can only detect BrdUrd incorporated into DNA when it is in ssDNA form. Raderschall et al. revealed these regions of damage in cells following treatment with DSB-inducing agents, such as etoposide and ionizing radiation (15–17). Their data suggested that ssDNA regions form immediately following DSB and that ssDNA regions form immediately following DSB and that ssDNA regions form immediately following DSB and that ssDNA regions form immediately following DSB and that ssDNA regions form immediately following DSB and that ssDNA regions form immediately following DSB. As noted previously, HDI have been shown to induce apoptosis in leukemic cells and this may be the underlying mechanism most important to the antitumor effects of these drugs (4). Consistent with this notion, although there was background apoptosis in our leukemic cell lines, using flow cytometry, we saw distinct G2-M arrest at 24 hours, and this was followed by a massive increase in a sub-G1 peak, indicating a profound apoptosis by 48 hours in our leukemic cell lines treated with HDI (data not shown). However, the onset of apoptosis, as marked by the cleavage of caspase-3 and poly(ADP-ribose) polymerase, begins as early as 4 hours after HDI administration in HL60 cells and well after the appearance of increased histone acetylation, activation of H2AX (Fig. 1A, C, and D; Supplementary Fig. S1A, B, and D), and ATM phosphorylation (Fig. 1E). Importantly, the DNA damage we observed in leukemic cells following HDI is not an early manifestation of apoptosis, because K562 cells stably transfected with antiapoptotic BCL2 (Fig. 2A) inhibit caspase cleavage (Fig. 2B and C) following HDI treatment but still induce DNA damage as measured by γH2AX (Fig. 2D).
induces histone acetylation along the chromatin fiber in treated compared with untreated cells (Fig. 3A), and this acetylation colocalizes with Ku86 (Fig. 3C). Furthermore, the HDI-induced phosphorylation of H2AX colocalizes with another nonhomologous end joining repair factor, DNA-PK (Fig. 3D). All of these changes also colocalize with the ssDNA regions revealed by BrdUrd staining along the length of the chromatin fiber (Fig. 3A and B). Importantly, to confirm that this assay can detect DNA damage, we used chromatin fibers from γ-irradiated HL60 cells as a positive control and similar DNA damage to that generated by HDI treatment that colocalizes with DNA-PK (Fig. 3E) was seen.

Importantly, in normal PBL treated with HDI, Western blot analyses for phosphorylated H2AX showed a significant increase in γH2AX protein phosphorylation in leukemic versus normal cells (Fig. 1B). Furthermore, we also saw evidence for DNA damage on chromatin fibers from normal PBL, but the amount of damage seemed considerably less than that induced in leukemic cells (Fig. 4A). The formation of γH2AX foci in nuclei following DNA damage has been used as a sensitive measure of DSB damage rather than measurement by Western blot analysis (18, 19). To assess the differences in DNA damage more quantitatively, we examined γH2AX foci formation in normal PBL and HL60 cells following HDI treatment. We showed that untreated HL60 cells contain low levels of γH2AX foci, whereas no foci are observed in normal PBL. HDI treatment results in a large increase in foci in HL60 cells compared with DNA damage in

FIGURE 1. DNA damage and apoptosis responses to treatment of cells with HDI as analyzed by Western blotting. A. Trichostatin A (TSA; 300 nmol/L) was added to HL60 cells for 24 hours. B. Trichostatin A (300 nmol/L) was added to PBL for 48 hours. C. Apicidin (1 μmol/L) was added to HL60 cells for 48 hours. D. Apicidin (1 μmol/L) added to primary cycling AML cells for 24 hours. E. ATM phosphorylation measured in HL60 cells treated with trichostatin A (300 nmol/L) for 480 minutes. F. HL60 cells were γ-irradiated and returned to culture for 480 minutes. G. Staurosporine (1 μmol/L) was added to HL60 cells for 24 hours. H. Staurosporine (1 μmol/L) was added to PBL for 48 hours.
DNA damage (7% from untreated cells versus 13% from treated cells; n = 3) than splenic APL cells from these mice. We were also able to show selective apoptosis of APL mice spleen cells with trichostatin A (Fig. 4D). Trichostatin A had little effect on normal FVB/N mouse caspase cleavage. FVB/N and APL cells treated with arsenic trioxide (As2O3) show apoptosis as shown previously (21-23). Thus, the amount of DNA damage and apoptosis were dramatically increased in leukemic versus normal cells with trichostatin A treatment in vivo as has been shown in vitro.

To determine whether there is a distinct functional link between the key initiating DNA damage repair factors and HDI, we examined leukemic cells in which expression of these proteins is abrogated. H2AX is an early chromatin modification that demarcates DSB (24, 25). We examined apoptosis and DNA damage in leukemia cell lines (K562, HL60, and NB4) silenced for H2AX following HDI treatment. In cells where H2AX expression is knocked down to ~20% of normal (Fig. 5A), there is a faster onset of caspase cleavage compared with HDI-treated, unsilenced counterparts. Thus, in apicidin- and trichostatin A–treated H2AX-silenced HL60 cells, caspase cleavage occurs at 120 minutes (Fig. 5B and C), whereas in HDI-treated control cells caspase cleavage is seen at 240 minutes (Fig. 1A and C). Early onset of apoptosis is also seen with sodium butyrate–treated, H2AX-silenced HL60 and K562 (Supplementary Fig. S2A and B) compared with unsilenced controls (Supplementary Fig. S1A and D). Furthermore, measurement of BrdUrd-positive ssDNA damage in HDI-treated leukemic cells silenced for H2AX showed that foci numbers are increased and seem to be sustained for a longer period of time compared with control small interfering RNA (siRNA)–treated cells (Fig. 5D; Table 3).

To confirm the altered DNA damage response following HDI treatment with γH2AX silencing, we examined cells null for another key early DNA damage response gene, ATM (GM5849). We find, as with H2AX silencing, that ATM-null cells show caspase cleavage at 2 hours following sodium butyrate treatment, whereas HDI-treated control fibroblasts show no apoptosis (GM00637; Fig. 5F and G). In addition, HDI-treated ATM−/− cells show increased γH2AX foci compared with control HDI-treated fibroblasts (Fig. 5E; Table 4).

Cancer cells exhibit defective checkpoints at all phases of the cell cycle (26). One mechanism that may explain the DNA damage that can occur through the alteration of chromatin structure or function induced by HDI treatment is if it occurs during DNA replication. If the DNA damage caused by HDIs is dependent on DNA replication, then wild-type cells because of intact DNA damage checkpoints might arrest in response to HDI-induced chromatin changes in S phase, thus showing no (or low levels of) DNA damage, whereas tumor cells might fail to effectively arrest and exhibit more DNA breakage. We therefore determined at which stage of the cell cycle DNA damage occurs in leukemic cells following HDI treatment using flow cytometry. Figure 6A shows that addition of 1 mmol/L sodium butyrate to HL60 for 30 minutes shows a predominant increase in γH2AX fluorescence in G1, whereas sodium butyrate treatment for 90 minutes shows a shift in
H2AX fluorescence toward G1-S, S, and S-G2 phases. In contrast, γ-irradiating HL60 cells results in a general increase in H2AX fluorescence at all stages of the cell cycle. Thus, HDI treatment does not result in DNA damage exclusively in S-phase cells.

**Discussion**

It is widely accepted that HDI evoke antitumor activity through enhancement of specific antitumor gene transcriptional activity. However, HDI only increase transcription of between 5% and 10% of genes, whereas no definitive

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antitumor gene activity has been attributed to HDI (27). It is thus conceivable that HDI exert their apoptotic properties by alternative means. We have shown here that in hematopoietic cell lines, primary cells, and mouse models a variety of HDI induce DNA damage and repair responses but result in significantly increased DNA damage in leukemic cells compared with normal cells. This damage occurs following induction of histone acetylation, and apoptosis occurs some time thereafter. This increased DNA damage in leukemic cells may well determine why they respond differentially to HDI with apoptosis compared with normal cells. Although the low levels of DNA damage in normal PBL are repaired by 1 hour after HDI treatment, DNA damage is still detectable in leukemic cells after 3 hours. Thus, leukemic cells may exceed a threshold of DNA damage that can be repaired, triggering apoptosis. Thus, our data are consistent with those observed by others where trichostatin A has been shown to induce apoptosis differentially in leukemic APL cells compared with normal cells (10, 11).

Our results suggest that this HDI-induced DNA damage may possibly occur through changes in chromatin structure. In fact, we show that phosphorylation of H2AX, one of the first cellular

FIGURE 4. Leukemia cells are more susceptible to trichostatin A–derived DNA damage than normal cells. HL60 and normal PBL cells were treated with trichostatin A (300 nmol/L) for 1 hour and fixed with formaldehyde and chromatin fibers were prepared. A. Coimmunostaining of chromatin fibers for DNA damage from HL60 and PBL cells grown for 30 hours in BrdUrd and treated with trichostatin A (30 minutes). Five randomly captured images of chromatin fibers were stained for acetylated histone H4 (red) and BrdUrd (green) and then merged; yellow, regions of colocalization of acetylated histone H4 DNA damage. B. Immunostaining of nuclei (DAPI; blue) from trichostatin A–treated HL60 and PBL cells for γH2AX foci (green).Cytopspins were prepared at the stated time points following incubation with trichostatin A and immunoprobed for γH2AX. Representative HL60 and PBL nuclei. C. Immunostaining of nuclei (DAPI; blue) of NB4 cells treated with trichostatin A, sodium butyrate, or apicidin (Api) for γH2AX foci (red). Cytopspins were prepared at the stated time points following incubation with HDI and immunoprobed for γH2AX. Representative NB4 nuclei. D. Caspase cleavage Western blot analysis of APL and FVB/N mouse cells treated previously with trichostatin A. Positive controls are mice treated with As2O3 (AS), which cause apoptosis in FVB/N and APL cells. Lane 1, FVB/N + As2O3; lane 2, FVB/N; lane 3, FVB/N + As2O3; lane 4, APL + As2O3; lane 5, APL + As2O3; lane 6, FVB/N + trichostatin A; lane 7, APL + trichostatin A; lane 8, APL + trichostatin A; lane 9, APL.
Table 1. Detection of Percentage of γH2AX Foci by Immunofluorescence following Trichostatin A Treatment

<table>
<thead>
<tr>
<th>Time point (min)</th>
<th>HL60</th>
<th>NB4</th>
<th>PBL</th>
<th>K562 + BCL2</th>
<th>K562 + pBABE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8 ± 1</td>
<td>6 ± 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
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<td>30</td>
<td>50 ± 5</td>
<td>55 ± 2</td>
<td>14 ± 6</td>
<td>32 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>60</td>
<td>41 ± 8</td>
<td>22 ± 6</td>
<td>9 ± 2</td>
<td>37 ± 8</td>
<td>3 ± 1</td>
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<td>180</td>
<td>22 ± 3</td>
<td>11 ± 5</td>
<td>0</td>
<td>27 ± 6</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE: One hundred nuclei were counted for each time point (n = 3).

Table 2. Detection of Percentage of γH2AX Foci in NB4 Cells by Immunofluorescence following Treatment with Sodium Butyrate and Apicidin

<table>
<thead>
<tr>
<th>Time point (min)</th>
<th>Sodium butyrate</th>
<th>Apicidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10 ± 2</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>10</td>
<td>60 ± 2</td>
<td>38 ± 3</td>
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<td>45 ± 3</td>
</tr>
<tr>
<td>180</td>
<td>10 ± 2</td>
<td>8 ± 1</td>
</tr>
</tbody>
</table>
cytogenetics analysis was made on each sample before primary cell harvesting using Hypaque-Ficoll gradients. The mononuclear fraction was isolated from 10 to 20 mL AML bone marrow aspirate. Cytospins of these fractions were examined morphologically after May-Grunwald Giemsa staining and revealed the presence of >95% of AML blasts (CD34+). Primary cells were cultured at 1 × 10⁶/mL in Iscove’s modified Dulbecco’s medium (Sigma-Aldrich) supplemented with 20% FCS, 4 mmol/L glutamine, and 1% penicillin/streptomycin. Cells were stimulated by adding 10 ng/mL stem cell factor (R&D Systems, Abingdon, United Kingdom), 10 ng/mL interleukin-6, and 10 ng/mL interleukin-3 and grown to a density of 1 × 10⁶/mL for 5 days. Log-phase cells were treated with HDIs at concentrations used previously to elicit histone acetylation. γ-Irradiation of log-phase cells was done on ice in a Gammarcell Cobalt 60 γ-iradiator at doses between 3 and 10 Gy (6.52 Gy/min). Cells were then returned to culture in fresh prewarmed medium for 10 minutes.

**Reagents**

Trichostatin A and sodium butyrate were purchased from Sigma-Aldrich and prepared in ethanol and PBS, respectively. Staurosporine, zVAD-fmk and apicidin (Calbiochem, Beeston, United Kingdom) were prepared in DMSO.

**Plasmids and Antibodies**

Rabbit polyclonal antisera raised against human and mouse Ku86, Ku70, DNA-dependent protein kinase catalytic subunit, and H2AX antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antisera against human and mouse Ku86, Ku70, DNA-dependent protein kinase catalytic subunit were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antisera against human and mouse Ku86, Ku70, DNA-dependent protein kinase catalytic subunit were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**FIGURE 5.** Effect of siRNA silencing of H2AX on HDI administration. A, Western blot analysis. Lane 1, untreated HL60 + H2AX siRNA; lane 2, γ-irradiated HL60 + H2AX siRNA; lane 3, trichostatin A–treated HL60 + H2AX siRNA; lane 4, γ-irradiated HL60 + scrambled siRNA; lane 5, trichostatin A–treated HL60 + scrambled siRNA. B and C, Western blot analysis of the effect of H2AX silencing on HDI-induced caspase cleavage in HL60: (B) apicidin for 72 hours and (C) trichostatin A for 72 hours. Lane +, 30-minute sample of γ-irradiated HL60. D, Immunostaining of nuclei (DAPI; blue) from sodium butyrate–treated, siRNA-silenced H2AX HL60 cells for BrdUrd-positive DNA damage foci (green). Cytospins were prepared at the stated time points following incubation with sodium butyrate and immunoprobed for BrdUrd, Representative HL60 nuclei. Sodium butyrate–treated HL60 were also transfected with scrambled siRNA to control for silencing. E, Immunostaining of nuclei (DAPI; blue) from sodium butyrate–treated, GM05849 (ATM−/−) fibroblasts and wild-type (WT) GM00637 fibroblasts for γH2AX foci (red). Representative nuclei. F and G, Western blot analysis of the effect of sodium butyrate on ATM−/− fibroblast and wild-type fibroblast caspase cleavage: (F) GM05849 (ATM−/−) for 72 hours and (G) GM00637 (wild-type) fibroblasts for 72 hours.
Table 3. Detection of Percentage of BrdUrd Foci in HL60 Cells by Immunofluorescence following siRNA Silencing of H2AX and Subsequent HDI Treatment

<table>
<thead>
<tr>
<th>Time point (min)</th>
<th>siRNA H2AX</th>
<th>siRNA Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>21 ± 3*</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>10</td>
<td>59 ± 6</td>
<td>49 ± 8</td>
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<td>20 ± 3</td>
</tr>
<tr>
<td>180</td>
<td>65 ± 2</td>
<td>7 ± 3</td>
</tr>
</tbody>
</table>

*Mean ± SD percentages of cells with BrdUrd foci (>5 foci per cell; n = 3).

caspase-3, poly(ADP-ribose) polymerase, tubulin, histone H4, and acetylated histone H4 were purchased from Serotec (Oxford, United Kingdom). Rabbit anti-γH2AX mouse anti-phosphorylated ATM (Ser1981) was purchased from Upstate (Charlottesville, VA). Mouse anti-human BCL2 was purchased from BD Biosciences (Franklin Lakes, NJ).

Retroviral Infection

pBABE-puro empty vector and pBABE-puro-BCL2 (35) cDNA constructs were used in the generation of replication-defective amphotropic retrovirus by transient transfection of the Phoenix-Ampho packaging line (American Type Culture Collection). This was subsequently used to infect parental K562 line as described previously (36). In brief, RetroNectin (Takara Biochemicals, Takara Shiga, Japan)—coated 24-well plate wells were blocked with 1% bovine serum albumin and infection was carried out with 400 μL retroviral supernatant from BD Biosciences (Franklin Lakes, NJ).

Western Blotting

Cell lysates (1 × 10⁶ cells) from leukemia myeloid cells, PBL, and primary CD34⁺ progenitor cells were prepared and resolved by SDS-PAGE (4-12% gradient gels; Invitrogen, Paisley, United Kingdom). Proteins were subsequently transferred to nitrocellulose membranes using electroblotting apparatus (Invitrogen). The membrane was blocked in 5% milk powder for 1 hour before overnight incubation at 4°C with primary antibody (1:1,000 dilution). Unbound antibody was removed with TBS containing 0.5% Tween 20. The membrane was then incubated with secondary antibody (horseradish peroxidase conjugated) for 1 hour at room temperature and washed four times in TBS containing 0.5% Tween 20, and detection was made using Enhanced Chemiluminescence Plus reagents (Amersham Pharmacia, Amersham, United Kingdom) according to the manufacturer’s instructions.

Chromatin Fibers

Chromatin fibers were prepared from 1 × 10⁶ cells according to the protocols of Raderschall et al. (15). However, the technique was modified to ensure subsequent detection of proteins binding at specific points of interest. Proteins were cross-linked to DNA by adding formaldehyde (1% final concentration) to the culture medium for 10 minutes at 37°C. Aliquots of 1 × 10⁶ cells were cytopsinto glass slides and covered with 50 μL of 50 mmol/L Tris-HCl (pH 8), 1 mmol/L EDTA, and 0.1% SDS. After 1-minute incubation with this detergent solution, the chromatin was mechanically sheared on the slide with the aid of a glass coverslip, fixed in ethanol for 30 minutes at −20°C, and then rinsed in ice-cold acetone for up to 1 minute.

Immunofluorescence

Slides with chromatin fibers were incubated with blocking solution (10% bovine serum albumin/4× SSC/0.1% Tween 20) for 30 minutes at 37°C. Thereafter, slides were incubated in primary antibody diluted in blocking serum (1:10-1:50) and incubated for 30 minutes at 37°C. Slides were washed for 5 minutes in 4× SSC/0.1% Tween 20 and this was repeated twice more. The blocking step was repeated before slides were incubated in secondary antibody conjugated with fluorochromes diluted in blocking solution (1:200; Sigma-Aldrich) and subsequently washed as above. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 1 minute and rinsed in PBS and coverslips were mounted in antifade solution ready for analysis. Slides were examined using an Olympus (London, United Kingdom) fluorescent microscope with DAPI/FITC/rhodamine triple-pass filters and images were captured using a charge-coupled camera and software (Smart capture VP, Digital Scientific Ltd., Cambridge, United Kingdom) and data were analysed (Quips XL, Vysis, Inc., Surrey, United Kingdom).

DNA Damage Studies

Cells were grown in 10 mmol/L BrdUrd (Sigma-Aldrich) for ~30 hours. Flasks were shielded from light. Thereafter, the cells were washed and placed in BrdUrd-free medium for 1 hour. Chromatin fibers were prepared from 1 × 10⁶ cells according to the protocols of Raderschall et al. (15). However, we modified the protocol to ensure subsequent detection of proteins binding at specific sites of interest. Proteins were cross-linked to DNA by adding formaldehyde (1% final concentration) to the culture medium for 10 minutes at 37°C before harvesting. Aliquots of 1 × 10⁶ cells were cytopsinto glass slides and covered with 50 μL of 50 mmol/L Tris-HCl (pH 8), 1 mmol/L EDTA, and 0.1% SDS. After 1-minute incubation with the detergent solution, the chromatin was mechanically sheared on the slide with the aid of a glass coverslip and then fixed with methanol and acetone. Antibodies to BrdUrd will only detect

Table 4. Detection of Percentage of γH2AX Foci by Immunofluorescence following Sodium Butyrate Treatment

<table>
<thead>
<tr>
<th>Time point (min)</th>
<th>ATM−/−/ GM05849</th>
<th>Wild-type control GM00637</th>
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<tbody>
<tr>
<td>0</td>
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<td>0</td>
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<td>10</td>
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<td>8 ± 1</td>
</tr>
<tr>
<td>180</td>
<td>21 ± 6</td>
<td>0</td>
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BrdUrd incorporation at ssDNA regions. Thus, this protocol is specific for the detection of regions of DNA damage.

**Immunofluorescence for Flow Cytometry**

After drug treatment, cells were fixed in 1% paraformaldehyde solution for 5 minutes at room temperature and then subsequently resuspended in 80% ethanol at −20°C overnight. Cells were then washed twice in wash solution (PBS/0.5% Tween 20/1% bovine serum albumin) and then incubated in 1% Triton X-100/10% bovine serum albumin for 30 minutes. Cells were washed again; thereafter, cells were resuspended in 100 μL anti-γH2AX diluted 1:50 in wash solution for 2 hours at room temperature. After further washings, the cells were then incubated in 100 μL anti-mouse IgG FITC conjugate (1:50) for 30 minutes at room temperature in the dark. Cells were counterstained with 40 μg/mL propidium iodide/1 mg/mL RNase for 30 minutes before fluorescence measurement. Green (FITC) and red (propidium iodide) fluorescence was measured using the appropriate filters on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). Experiments were done in duplicate in at least three different experiments.

**siRNA Silencing**

siRNA duplexes were manufactured by Dharmacon (Dallas, TX). The sense strand for human H2AX was CAACAAGAA-GACGCGAAUCdTdT. A control siRNA was synthesized with no complementarity to H2AX. Transfection of myeloid cell lines with siRNA was carried out using Oligofectamine (Invitrogen) according to the manufacturer’s instructions. Cells (1 × 10⁶) in 15 mL Opti-MEM, including 10% FCS (Invitrogen) per T75 culture flask (Sarstedt, Leicester, United Kingdom), was incubated 24 hours before transfection. siRNA (10 nmol/L) was added to 25 μL Oligofectamine and added to the cells in 4 mL fresh Opti-MEM. Four hours after transfection, 2.5 mL Opti-MEM containing 30% FCS was added to the flask. Seventy-two hours after transfection, cultures were subjected to irradiation (3 Gy) and returned to culture for 15 minutes or 300 nmol/L trichostatin A was added for 2 hours. Cells were then harvested for Western blot analysis. Multiple siRNA cultures were set up to make nuclear extracts.

**Cell Cycle Analysis**

HDI were added to log-phase cells for 72 hours. At the indicated times, 2 × 10⁵ cell aliquots were taken and centrifuged at 400 × g for 10 minutes. The supernatant was aspirated and 70% ethanol (at −20°C) was added and gently vortexed. The cell suspension was again centrifuged at 400 × g for 10 minutes. The supernatant was aspirated and 1 mL staining solution (PBS + 5 ng FITC, 40 μg propidium iodide, 500 μg RNase) was added. Samples were analyzed using the FACSscan flow cytometer (Becton Dickinson). To identify S-phase cells, log-phase cultures were pulsed with 10 mmol/L BrdUrd for 30 minutes before preparation of cytopsin followed by denaturing DNA and immunostaining as described previously.

**Animal Models**

Transgenic mice using the human PML-retinoic acid receptor α cDNA were constructed previously in the FVB/N inbred strain of mice (20). This transgene contains a new amino acid generated by the fusion of PML with retinoic acid receptor α. The transplant model is estimated from 10⁶ blast cells isolated from spleens of transgenic animals injected i.v. in 6- to 8-week-old naive syngeneic mice (20), which was the minimum dose sufficient to induce leukemia in ~3 weeks. Establishment of leukemia is assessed by the appearance of high leukocyte and low platelet...
counts at around day 10 (ref. 20; Hemavet counter, CDC Technologies, Oxford, CT). Enlargement of organs and tissue section examination confirms the cause of death from APL (21). All procedures complied with European or national regulations. On day 14 after APL injection, trichostatin A (1 mg/kg) was given i.p. for 4 hours; on day 11 after APL injection, trichostatin A was given for 4 days and 4 hours was found to be sufficient to induce maximal misrepair. Spleen cells were harvested and assayed for misrepair and DNA damage after trichostatin A administration for 4 hours and for apoptosis after trichostatin A administration for 4 days. As positive controls, for apoptosis, mice were treated with As2O3. A stock solution of 330 mmol/L As2O3 was prepared by diluting the powder in 1 mol/L NaOH; then, a dilution in TBS was given by daily i.p. injection at the concentration of 5 μg/mice for a period of 5 days.

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References

Histone Deacetylase Inhibitors (HDI) Cause DNA Damage in Leukemia Cells: A Mechanism for Leukemia-Specific HDI-Dependent Apoptosis?

Terry J. Gaymes, Rose Ann Padua, Marika Pla, et al.


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