High DNA Methyltransferase 3B Expression Mediates 5-Aza-Deoxycytidine Hypersensitivity in Testicular Germ Cell Tumors

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Abstract

Testicular germ cell tumors (TGCT) are the most common solid tumors of 15- to 35-year-old men. TGCT patients are frequently cured with cytotoxic cisplatin-based therapy. However, TGCT patients refractory to cisplatin-based chemotherapy have a poor prognosis, as do those having a late relapse. Pluripotent embryonal carcinomas (EC) are the malignant counterparts to embryonic stem cells and are considered the stem cells of TGCTs. Here, we show that human EC cells are highly sensitive to 5-aza-deoxycytidine (5-aza-CdR) compared with somatic solid tumor cells. Decreased proliferation and survival with low nanomolar concentrations of 5-aza-CdR is associated with ATM activation, H2AX phosphorylation, increased expression of p21, and the induction of genes known to be methylated in TGCTs (MGMT, RASSF1A, and HOXAX). Notably, 5-aza-CdR hypersensitivity is associated with markedly abundant expression of the pluripotency-associated DNA methyltransferase 3B (DNMT3B) compared with somatic tumor cells. Knockdown of DNMT3B in EC cells results in substantial resistance to 5-aza-CdR, strongly indicating that 5-aza-CdR sensitivity is mechanistically linked to high levels of DNMT3B. Intriguingly, cisplatin-resistant EC cells retain an exquisite sensitivity to low-dose 5-aza-CdR treatment, and pretreatment of 5-aza-CdR resensitizes these cells to cisplatin-mediated toxicity. This resensitization is also partially dependent on high expression of DNMT3B, at which 5-aza-CdR hypermethylates differentiation. These novel findings indicate that high expression of DNMT3B, a likely byproduct of their pluripotency and germl cell origin, sensitizes TGCT-derived EC cells to low-dose 5-aza-CdR treatment. [Cancer Res 2009;69(24):OF1–7]

Introduction

Testicular germ cell tumors (TGCT), the most common solid tumors of adolescent and young men, are thought to derive from transformation of primordial germ cells (PGC) or early gonocytes (1, 2). TGCTs are classified as seminomas and nonseminomas (1). Nonseminomas are undifferentiated, pluripotent cells, known as embryonal carcinoma (EC). ECs are proposed to represent key features of this human malignancy (1). Mechanisms of inherent or acquired cisplatin resistance in other tumors have not yielded insights into the exquisite cisplatin sensitivity of TGCTs (4). That patients with advanced stage TGCTs can be cured implies that the stem cells of TGCTs are effectively targeted with cisplatin-based chemotherapy (1, 4).

To date, DNA methylation inhibitors have been more active in leukemia than in solid tumor cells (7). There is currently little information available on the effects of DNA methylation inhibitors against TGCT cells. In the current study, we establish that TGCT cells are hypersensitive to the DNA methylation inhibitor 5-aza-deoxycytidine (5-aza-CdR). This response is associated with remarkably high levels of DNA methyltransferase 3B (DNMT3B). Notably, high DNMT3B expression is validated as functionally important for 5-aza-CdR hypersensitivity in both cisplatin-sensitive and cisplatin-resistant TGCT cells. Indeed, 5-aza-CdR can resensitize cisplatin-resistant cells to cisplatin-mediated toxicity. Together, these findings indicate that high basal DNMT3B expression in pluripotent EC cells can account for 5-aza-CdR hypersensitivity in cisplatin-sensitive and cisplatin-resistant TGCTs.

Materials and Methods

Cell culture and drug treatments. All cell lines were cultured in DMEM media with 10% fetal bovine serum supplemented with glutamine and antibiotics with the exception of MCF7 cells that were cultured in F12-DMEM. The derivation of the NT2/DI-resistant NT2/D1-R1 cell line was previously described (8, 9). The expression of results in Fig. 4D was performed on 1 µg RNA using the Taqman RT kit (Applied Biosystems). Twenty nanograms of the resulting cDNA was used with SYBR green (Applied Biosystems) for quantitative real-time PCR assays using the ddCT

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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method normalized to glyceraldehyde-3-phosphate dehydrogenase and the ABI Prism Sequence Detection System 7700. Primers are provided in Supplementary Table S1. For Western analyses, cells were lysed in a radiimmune precipitation buffer and separated by SDS-PAGE, as previously described (8, 9). Antibodies to DNMT3B (H-230; sc-20704, Santa Cruz, and Ab2851, Abcam), actin (C-11; sc01615, Santa Cruz), 1981-ATM (Epitomics), and 139-H2AX (Cell Signaling) were used.

Lentiviral production. Silencing shRNAs targeting human DNMT3B were purchased (Open Biosystems). A plKO.1-shRNA lentiviral construct was also purchased (plKO.1, Sigma) and used as a control. Lentiviral stocks were generated from 293T cells, and psPAX2 and pMD2G packaging and was also purchased (pLKO.1, Sigma) and used as a control. Lentiviral stocks were purchased (Open Biosystems). A pLKO.1-shRNA lentiviral construct.

Results

EC sensitivity to low-dose 5-aza-CdR. We sought to identify whether different EC cell lines were sensitive to DNA methylation inhibition. Five different EC cell lines, including two that were cisplatin resistant, NT2/D1-R1 and 833K-CP, compared with parental NT2/D1 and 833K lines, were highly sensitive to inhibition of cell growth and viability with the DNA methylation inhibitor 5-aza-CdR with IC98 in the 5 to 25 nmol/L range (Fig. 1). These doses are substantially lower than those typically reported for diverse solid somatic tumors that have IC98 in the 500 nmol/L to 10 μmol/L range (7, 10). This is in agreement with our somatic tumor cell data as human breast (MCF7), osteosarcoma (U2OS), and colon (HCT116) cancer cells were relatively resistant to 5-aza-CdR treatment at doses as high as 5 μmol/L (Fig. 1). Interestingly, the line most sensitive to 5-aza-CdR was the cisplatin-resistant line, 833K-CP (also called 833K64-CP10).

EC cells overexpress DNMT3B compared with somatic cancer cells. Recent microarray studies indicated that ES and EC cells as well as clinical EC and nonseminomas highly express mRNA for the DNA methyltransferase, DNMT3B, compared with normal and somatic tumors (11–13). However, this differential expression has not been confirmed or shown at the protein level. We found a striking difference in DNMT3B protein expression in the EC cell lines NT2/D1, NT2/D1-R1, 833K, 833K-CP, Tera-1, 577M, and 2102EP compared with U2OS, MCF7, and lung, pancreatic and glioblastoma cancer cell lines (Fig. 2). HCT116 cells also did not express appreciable DNMT3B compared with EC cells (data not shown). Notably, the high expression of DNMT3B in EC cells could be detected with two distinct DNMT3B antibodies (Fig. 2). Densitometry measurements revealed at least a 30-fold increase in DNMT3B expression in the EC lines compared with somatic tumors. Thus, the hypersensitivity of TGCTs to low-dose 5-aza-CdR is tightly associated with high expression of DNMT3B in EC cells. It should be noted that there is not an exact correlation between the EC cell lines with regard to the level of DNMT3B and sensitivity to 5-aza-CdR. This is most evident in the NT2/D1-R1 line that is less sensitive to 10 nmol/L 5-aza-CdR compared with other EC cells, yet is one of the highest expressers of DNMT3B. Thus, there seems to be other cell line–specific factors.

Statistics. Where a value for statistical significance is indicated, a two sample two-tailed t test assuming unequal variances was performed.
were independently used to knockdown DNMT3B. Six potential
hypersensitivity.
and the induction of genes known to be methylated in TGCTs.
low doses of 5-aza-CdR can elicit both a DNA damage response
in response to DNA damage (22). Together, the data suggest that
be commonly methylated in tumor cells but is commonly induced
able to induce the expression of p21. The p21 gene is not known to
not induced by low-dose 5-aza-CdR. Low-dose 5-aza-CdR was also
dose 5-aza-CdR induced the expression of MGMT, RASSF1A, and
RASSF1A, HOXA9, and BRCA1 (19
of genes known to be methylated in TGCTs, expression of MGMT,
ment of NT2/D1-R1 cells increased the levels of activated ATM and
these mechanisms at the low doses affecting cell proliferation
re-expression of tumor suppressor genes through DNA de-
amal protocol for 5-aza-CdR in the treatment of leukemia (15).
DNMT3B expression on the sensitivity of NT2/D1-R1 cells to chronic 5-aza-CdR treatment was assessed. NT2/D1-R1 cells and control shRNA cells showed an even greater sensitivity to a prolonged 10-day treatment with 5-aza-CdR compared with the 3-day treatment (Fig. 4D). These cells were sensitive to 5-aza-CdR at doses as low as 2.5 nmol/L. In contrast, NT2/D1-R1 cells treated with DNMT3B-targeting shRNA showed a dramatic resistance to 5-aza-CdR (Fig. 4D).

Restoring cisplatin sensitivity to resistant cells with 5-aza-
Cdr. We previously reported that cisplatin causes a global p53-
dominant transcriptional response in EC cells (23). Through microarray and other studies, we found that the p53 response is repressed in NT2/D1-R1 cells despite having abundant wild-type p53 expression (data not shown; refs. 8, 9, 23). Figure 5A shows that pretreatment of NT2/D1-R1 cells with low-dose (10 nmol/L) 5-aza-CdR for 3 days at least partially restored cisplatin induction of the p53 target genes GDF15 and BTG2 in NT2/D1-R1 cells. Notably, 5-aza-CdR treatment alone substantially induced the expression of GDF15, BTG2, and the p53 target gene, FDXR, in these cells. In contrast, 5-aza-CdR treatment had little or no detected effect on cisplatin induction of FDXR in NT2/D1-R1 cells. This dose of 5-aza-CdR inhibits proliferation of NT2/D1-R1 cells by only ~10% versus controls after 3 days (Fig. 1). Viable cells were counted and replated after 5-aza-CdR treatment and allowed to recover for 24 hours before cisplatin treatment. Notably, pretreatment with low-dose 5-aza-CdR restored cisplatin growth suppression and toxicity to two separate cisplatin-resistant TGCT cell lines, NT2/D1-R1 and 833K-CP (Fig. 5B and C). 833K-CP cells were pretreated with 2.5 nmol/L 5-aza-CdR, a dose that results in a 10% growth inhibition (Fig. 1). Pretreatment with 5-aza-CdR did not alter the cisplatin sensitivity of parental NT2/D1 cells, but did partially increase the cisplatin sensitivity of 833K cells (data not shown). These data indicated that 5-aza-CdR can restore cisplatin cytotoxic response to cisplatin-resistant EC cells.

Relationship between DNMT3B levels and cisplatin sensitivity. Because DNMT3B knockdown reversed 5-aza-CdR hypersensitivity in cisplatin-sensitive and cisplatin-resistant EC cells, it was of interest to investigate whether cisplatin sensitivity itself depended on DNMT3B expression. As shown in Fig. 6, DNMT3B knockdown had no appreciable effect on cisplatin sensitivity of the cisplatin-resistant EC line NT2/D1-R1. This is in agreement with the finding
other than DNMT3B, which affect the relative sensitivity of the EC cells to 5-aza-CdR.

Low-dose 5-aza-CdR induces H2AX and ATM phosphorylation and tumor suppressor gene expression. Two mechanisms have been proposed to account for the antitumor effects of 5-aza-CdR, namely, activation of the DNA damage response pathway and re-expression of tumor suppressor genes through DNA de-
methylation (14–18). We asked whether 5-aza-CdR engaged these mechanisms at the low doses affecting cell proliferation and survival of EC. As shown in Fig. 3A, low-dose 5-aza-CdR treatment of NT2/D1-R1 cells increased the levels of activated ATM and phosphorylated H2AX, two hallmarks of the DNA damage response previously shown to be induced with high-dose 5-aza-CdR in somatic tumor cells (7, 17, 18).

To address whether low-dose 5-aza-CdR induced the expression of genes known to be methylated in TGCTs, expression of MGMT, RASSF1A, HOXA9, and BRCA1 (19–21) was assessed (Fig. 3B). Low-dose 5-aza-CdR-induced expression of MGMT, RASSF1A, and HOXA9 in NT2/D1-R1 cells. In contrast, expression of BRCA1 was not induced by low-dose 5-aza-CdR. Low-dose 5-aza-CdR was also able to induce the expression of p21. The p21 gene is not known to be commonly methylated in tumor cells but is commonly induced in response to DNA damage (22). Together, the data suggest that low doses of 5-aza-CdR can elicit both a DNA damage response and the induction of genes known to be methylated in TGCTs.

Knockdown of DNMT3B in EC cells reverses 5-aza-CdR hypersensitivity. Five different lentiviral shRNAs for DNMT3B were independently used to knockdown DNMT3B. Six potential alternatively spliced isoforms of DNMT3B exist; the most biologically relevant isoforms are variants 1, 2, 3, and 6 (14). Quantitative reverse transcription-PCR assays using isoform-specific primers revealed that these shRNAs (relative to controls) reduced expression of these DNMT3B isoforms (Fig. 4A). Furthermore, none of the DNMT3B-specific shRNAs affected levels of DNMT1 or DNMT3A (Fig. 4A). DNMT3B-targeting shRNAs also reduced DNMT3B protein in both NT2/D1 and NT2/D1-R1 cells (Fig. 4B). Because NT2/D1 cells stably expressing sh84 and NT2/D1-R1 cells stably expressing sh84, sh85, or sh86 had the most efficient knockdown of DNMT3B (Fig. 4A and B), these cells were tested for 5-aza-CdR sensitivity. Cells expressing DNMT3B-targeting shRNAs exhibited dramatic reduction of 5-aza-CdR sensitivity compared with control cells (Fig. 4C). However, knockdown of DNMT3B by itself had no apparent effect on the growth of NT2/D1 or NT2/D1-R1 cells (data not shown). These results strongly support a functional link between sensitivity of EC cells to 5-aza-CdR and high DNMT3B expression.

Chronic low-dose scheduling has been suggested to be the optimal protocol for 5-aza-CdR in the treatment of leukemia (15). Therefore, the effect of DNMT3B expression on the sensitivity of NT2/D1-R1 cells to chronic 5-aza-CdR treatment was assessed. NT2/D1-R1 cells and control shRNA cells showed an even greater sensitivity to a prolonged 10-day treatment with 5-aza-CdR compared with the 3-day treatment (Fig. 4D). These cell lines were sensitive to 5-aza-CdR at doses as low as 2.5 nmol/L. In contrast, NT2/D1-R1 cells treated with DNMT3B-targeting shRNA showed a dramatic resistance to 5-aza-CdR (Fig. 4D).

Figure 3. Low-dose 5-aza-CdR activates the ATM pathway and induces the expression of methylated genes in EC cells. A, indicated doses of 5-aza were added fresh each day for 3 d to NT2/D1-R1 cells. Expression profiles of P(S198) ATM and P(S139)H2AX were assessed. The experiment was repeated with similar results. B, NT2/D1-R1 cells were treated as in A above and expression of the indicated genes assessed by quantitative PCR assays. Columns, mean of biological triplicate determinations; bars, SD; *, P < 0.01; #, P < 0.05.
that both cisplatin-sensitive (NT2/D1 and 833K) and cisplatin-resistant (NT2/D1-R1 and 833K-CP) EC cells abundantly express DNMT3B (Fig. 2). However, the ability of 5-aza-CdR to sensitize NT2/D1-R1 cells to cisplatin was substantially and significantly repressed by DNMT3B depletion. In summary, these data indicate that 5-aza-CdR hypersensitivity of EC cells is dependent on DNMT3B expression regardless of whether the cells are sensitive or resistant to cisplatin. Yet, cisplatin sensitivity per se is not affected by DNMT3B expression.

Discussion

Few studies have addressed 5-aza cytidine analogue treatment effects in TGCT cells. In the current study, we show that TGCT cells are hypersensitive to the DNA methylation inhibitor, 5-aza-CdR. This response is tightly associated with high levels of DNMT3B protein, which was validated as an important target of 5-aza-CdR–mediated hypersensitivity in cisplatin-sensitive as well as cisplatin-resistant TGCTs. Based on these findings, we propose that TGCT cells may be distinctly sensitive to DNA methylation inhibitors due to high basal levels of DNMT3B that are likely a consequence of the PGC origins of EC and their similarities to ES cells, which also are known to express high levels of DNMT3B (11, 12).

The 5-aza cytidine–related compounds become incorporated into DNA and have been shown to mediate covalent adduct formation with DNMTs (16). The most widely studied and accepted mechanism for the antitumor effects of 5-aza-CdR is related to DNA demethylation and re-expression of specific tumor suppressor genes (reviewed in ref. 14). However, other studies indicate that DNA methylation–independent effects of 5-aza-CdR are critical, especially for effects mediated through the ATM/ATR/p53-dependent DNA damage checkpoint (7, 16–18, 24, 25). The degree each mechanism is used may be cell context–dependent. The studies cited above use doses of 5-aza considerably higher than the doses used here in the treatment of EC cells. We provide evidence that in EC cells, low-dose 5-aza-CdR is able to activate the ATM pathway as well as induce the expression of genes known to be methylated in TGCTs (Fig. 3).
At first glance, it may seem paradoxical that EC cells are hypersensitive to a DNA methylation inhibitor, but are resistant to that inhibitor when one of its targets, DNMT3B, is depleted (Fig. 4). This result argues that the actions of 5-aza-CdR in EC cells cannot be explained fully by inhibition of DNMT3B activity and suggests that high basal levels of DNMT3B are needed to efficiently elicit an acute cytotoxic response to 5-aza-CdR.

This finding is not without precedence. Juttermann and colleagues (16) showed that ES cells and embryos with DNMT1 knockdown were significantly less sensitive to 5-aza-CdR-mediated toxicity. Oka and colleagues (26), using DNMT3B/DNMT3A null ES cells, also showed that DNMT3B/DNMT3A knockdown results in decreased sensitivity to 5-aza-CdR. HCT116 cells that contain a hypomorphic, truncated allele of DNMT1 were shown to have less...
The toxic effects of DNMT3B appear not to be engaged by general DNA-demethylating agents, as both cisplatin-sensitive and cisplatin-resistant cells were pretreated with vehicle or 5-aza-CdR (10 nmol/L) for 3 d before replating and a 24-h recovery period followed by the indicated cisplatin treatments for 6 h. Cell viability was assayed 3 d later via the Cell-Titer Glo assay. Points, mean of biological triplicates; bars, SD; N.s., no significant difference in cisplatin resistance ($P > 0.05$) for control shRNA cells versus shRNA-84 and shRNA-86 cells; *, significant change ($P < 0.02$) for 5-aza-CdR pretreated control shRNA cells versus 5-aza-CdR pretreated shRNA-84 and shRNA-86 cells. The experiment was independently repeated with similar results (data not shown).

H2AX phosphorylation and ATM activation in response to 5-aza-CdR, compared with wild-type HCT116 cells (17). However, cells with the hypomorphic DNMT1 allele were sensitized to 5-aza-CdR cytotoxicity, presumably due to a failure to effectively arrest in G2-M (17). In contrast, our preliminary data have thus far not detected large differences in 5-aza-CdR–mediated ATM and H2AX activation in DNMT3B knockdown cells, compared with wild-type EC cells (data not shown). As DNMTs are auxiliary components of the DNA replication and repair machinery (17, 27), it is possible that depleting DNMT3B in the EC context alters downstream responses to 5-aza-CdR–mediated DNA damage that perturbs the balance between cell cycle checkpoint arrest, DNA repair, and apoptosis.

In contrast to 5-aza-CdR sensitivity, cisplatin sensitivity is independent of DNMT3B levels, as both cisplatin-sensitive and cisplatin-resistant cells express abundant DNMT3B (Fig. 2) and knockdown of their pluripotent and germ cell origin, and results in hypersensitivity to DNA methylation inhibitors. The finding that cisplatin-resistant EC cells retain a high degree of sensitivity to low-dose 5-aza-CdR and that pretreatment of 5-aza-CdR restores cisplatin cytotoxicity to resistant EC cells is notable because it can be clinically exploited.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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