Inhibition of DNA Methyltransferase Activity Prevents Tumorigenesis in a Mouse Model of Prostate Cancer

Michael T. McCabe, Jonathan A. Low, Stephanie Daignault, Michael J. Imperiale, Kirk J. Wojno, and Mark L. Day

Departments of Urology and Microbiology and Immunology and Program in Cellular and Molecular Biology, University of Michigan, Ann Arbor, Michigan

Abstract

Transcriptional silencing of tumor suppressor genes by DNA methylation plays an important role in tumorigenesis. These aberrant epigenetic modifications may be mediated in part by elevated DNA methyltransferase levels. DNA methyltransferase 1 (DNMT1), in particular, is overexpressed in many tumor types. Recently, we showed that Dnmt1 is transcriptionally regulated by E2F transcription factors and that retinoblastoma protein (pRb) inactivation induces Dnmt1. Based on these observations, we investigated regulation of Dnmt1 by polyomavirus oncocenes, which potently inhibit the pRb pocket protein family. Infection of primary human prostate epithelial cells with BK polyomavirus dramatically induced Dnmt1 transcription following large T antigen (Tag) translation and E2F activation. For in vivo study of Dnmt1 regulation, we used the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, which expresses the SV40 polyomavirus early region, including Tag, under control of a prostate-specific promoter. Analysis of TRAMP prostate lesions revealed greatly elevated Dnmt1 mRNA and protein levels beginning in prostatic intraepithelial neoplasia and continuing through advanced prostate cancer and metastasis. Interestingly, when TRAMP mice were treated in a chemopreventive manner with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza), 0 of 14 mice developed prostate cancer at 24 weeks of age, whereas 7 of 13 (54%) control-treated mice developed poorly differentiated prostate cancer. Treatment with 5-aza also prevented the development of lymph node metastases and dramatically extended survival compared with control-treated mice. Taken together, these data suggest that Dnmt1 is rapidly activated by pRb pathway inactivation, and that DNA methyltransferase activity is required for malignant transformation and tumorigenesis.

Introduction

Epigenetic silencing of tumor suppressor genes by DNA hypermethylation is believed to play a major role in the etiology of many cancers. Prostate cancer, in particular, frequently exhibits methyltransferase activity is required for malignant transformation and tumorigenesis. These gene loci lead to a heritable transcriptional silencing, which is believed to be critical to tumorigenic progression.

DNA methylation is mediated by a family of three DNA methyltransferase enzymes: DNMT1, DNMT3a, and DNMT3b. Protein and activity levels of DNMT1 are elevated in prostate cancer compared with benign prostatic hyperplasia (5). DNMT1 is also elevated in other cancers, including gastric cancer (6), acute myelogenous leukemia (7), and cancers of the bladder, kidney, and colon (8). Experimental models have shown that overexpression of Dnmt1 in mouse embryonic stem cells is capable of inducing genomic DNA hypermethylation and loss of imprinting similar to that observed in many cancers (9). Furthermore, exogenous Dnmt1 expression in murine and human fibroblasts promoted many features of transformation, including spindle morphology, decreased contact inhibition, increased genomic methylation, increased growth in soft agar, and increased tumorigenicity (10–12).

Conversely, down-regulation of DNA methyltransferase activity has also been shown to have dramatic effects on transformation and tumorigenesis in several model systems. Despite embryonic lethality of the homozygous Dnmt1 knockout, hypomorphic Dnmt1 alleles that express 50% of normal DNA methyltransferase activity have been successfully created (13, 14). Despite the considerable decrease in DNA methyltransferase levels and activity, these animals remain viable and seem to maintain near normal levels of genomic DNA methylation (14). Interestingly, these hypomorphic Dnmt1 alleles significantly decreased tumor formation when introduced into the mismatch repair deficient MLH1−/− and colon cancer prone Apetl−/− mice models (13, 15, 16). Another model of carcinogen-induced lung cancer showed a 47% reduction in hyperplastic and neoplastic lesions by inactivating one allele of Dnmt1 (17). When Dnmt1−/− mice were further treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza) to inhibit remaining DNA methyltransferase activity, lesion formation was decreased by 89% compared with untreated wild-type mice (17). Taken together, these in vivo findings strongly suggest that DNA methyltransferase activity plays a necessary role in tumorigenesis. Therefore, DNMT1 seems to present itself as an alluring therapeutic target as it is overexpressed in cancer, its down-regulation decreases cancer incidence in mouse models, and specific inhibitors of the enzyme currently exist.

We and others have recently shown that the mouse and human DNMT1 gene promoters contains E2F-binding sites near the transcription initiation point, and that these genes are transcriptionally activated by E2F1 (18, 19). Furthermore, cells lacking the retinoblastoma gene (Rb1) expressed elevated Dnmt1 levels due to aberrant E2F activity (19). The loss of E2F control in Rb6−/− cells led to aberrant cell cycle-independent regulation of Dnmt1 and hypermethylation of a tumor suppressor gene (19). Additionally, we have shown in a separate model that infection of primary human kidney cells with human polyomavirus BKV induced E2F...
activity in response to viral large T antigen (Tag) expression and pRb inactivation. In this model, Dnmt1 was potently up-regulated, and this activation was dependent upon pRb inactivation and E2F binding of the Dnmt1 promoter. These findings suggest that pRb pathway inactivation, which is nearly ubiquitous in cancer, may play an important role in the initiation of abnormal DNA methylation patterns due to Dnmt1 overexpression.

The transgenic adenocarcinoma of the mouse prostate (TRAMP) model uses a transgene encoding the early region (Tag and Tag) of the SV40 polyomavirus under control of the rat probasin promoter (20). The probasin promoter allows for temporal and spatial regulation of transgene expression within the ventral and dorsal prostate lobes of sexually mature male mice (20). This mouse model undergoes a well-defined progression from normal prostate (before 6 weeks of age) to hyperplasia, adenocarcinoma, metastasis, and death by 40 weeks of age. Based upon the data supporting a role for DNA hypermethylation and DNA methyltransferase over-expression in tumorigenesis, we hypothesized that inhibition of DNA methyltransferase activity with 5-aza-deoxycytidine would decrease prostate tumorigenesis in the TRAMP model.

Materials and Methods

Cell cultures and viral infection. Primary human prostate epithelial cells (Clonetics, Mountain View, CA) from two individual donors were propagated in RPMI 1640 (Bio Whittaker, Walkersville, MD) supplemented with 0.01 mg/mL bovine pituitary extract (Sigma, St. Louis, MO), 0.5 μg/mL cholera toxin (Sigma), 2 μmol/L dexamethasone (Sigma), 5 ng/mL insulin-like growth factor-1 (Collaborative Research, Bedford, MA), 10 ng/mL epidermal growth factor (Collaborative Research), penicillin/streptomycin (Life Technologies, Grand Island, NY), 2 mmol/L l-glutamine (Life Technologies), and 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL selenious acid in an insulin, transferrin, and selenium premix (Collaborative Research). For viral infection, human prostate epithelial cell were grown in six-well dishes to 70% confluence and infected with BKV at a multiplicity of infection of 5 focus-forming units per cell as previously described (21).

RNA isolation and reverse transcription-PCR. Total cellular RNA was extracted from ~5 × 10^6 cells or 30-mg tissue with Qiagen’s RNeasy Mini kit (Valencia, CA) according to the manufacturer’s directions. Two micrograms of the resulting total RNA were reverse transcribed using the Thermoscript Reverse Transcription-PCR (RT-PCR) system (Invitrogen, Carlsbad, CA) as previously described (19). Variable numbers of PCR cycles were done to ensure analysis of samples during the linear phase of amplification. Gene-specific primer sequences are available upon request.

Protein isolation and Western blotting. Cells were harvested by mechanical disruption with cell scrapers followed by gentle centrifugation. Cell pellets were then lysed in appropriate volumes of lysis buffer [50 mmol/L Tris (pH 8), 120 mmol/L NaCl, 0.5% NP40, 1 mmol/L EDTA, 100 μg/mL phenylmethylsulfonyl fluoride, 50 μg/mL aprotinin, 50 μg/mL leupeptin, and 1.0 mmol/L sodium orthovanadate] for 1 hour on ice. Tissues were washed in PBS and homogenized in lysis buffer on ice for 20 seconds with a Tissue Tearer. Cellular debris was then pelleted by centrifugation, and supernatants were collected and quantitated using a Bradford protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein were then separated on precast Tris-glycine SDS-polyacrylamide gels (Novex, Carlsbad, CA) and transferred to Hybond nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were then blocked, probed, and developed as previously described (19). Primary antibodies were obtained as follows: actin (Santa Cruz Biotechnology, Santa Cruz, CA), Dnmt1 (Santa Cruz Biotechnology), proliferating cell nuclear antigen (PCNA, Santa Cruz Biotechnology), pRb (PharMingen, San Diego, CA), and large Tag (PA616; refs. 22, 23).

Mice. Male C57BL/6 mice hemizygous for the pRb-Tag transgene (derived from TRAMP founder B247; ref. 20) were mated with wild-type female C57BL/6 mice (Charles River, Wilmington, MA) to generate both wild-type and hemizygous TRAMP pups. All mice were maintained under specific pathogen-free conditions with ad libitum food and water in University of Michigan animal housing facilities.

Genotyping. Tail tissue (3 mm) obtained from 15-day-old pups was incubated overnight at 57°C in 600 μL TNES [10 mmol/L Tris (pH 7.5), 400 mmol/L NaCl, 100 mmol/L EDTA, and 0.6% SDS] and 35 μL proteinase K (10 mg/mL). Following the addition of NaCl to 1.25 mol/L, samples were centrifuged, and genomic DNA was precipitated from the supernatant with 1 volume 100% ethanol. DNA was then spotted onto a closed-end capillary tube, washed thrice in 70% ethanol, and dissolved in TE buffer. The resulting DNA was used in a genotyping PCR reaction with the following primers: Tag forward 5′-CCGGTCGACCGAAGCTTCCAAGTGCATTITA-3′ and Tag reverse 5′-AGGGATTCACCCACTGTCCTCATTAC-3′.

Maximum tolerable dose determination. Based on existing literature, a range of 5-aza doses (0.25-2.5 mg/kg) and treatment frequencies (two or three times weekly) were tested by i.p. injection in postpubertal wild-type male C57BL/6 mice for toxicity over a maximum 18-week treatment period. Toxicity was primarily detected as significant acute weight loss but also as altered gait, lethargy, ruffled coat, and hindered breathing.

Treatments. At 6 weeks of age, mice were randomized to receive either control (PBS) or 5-aza (Sigma). Treatments were administered twice weekly on consecutive days as 300 μL i.p. injections of either PBS or 0.25 mg/kg 5-aza dissolved in PBS. Treatment was continued until 24 weeks of age when mice were left untreated until they died spontaneously or exhibited signs of marked morbidity, including, but not limited to, progressive weight loss, altered gait, or significant lethargy.

Necropsy, tissue procurement, and statistical analysis. Mice were weighed and euthanized at 16 weeks, 24 weeks, or upon signs of morbidity by CO2 inhalation followed by induction of a bilateral pneumothorax. An incision was created along the midline from the pelvis to above the rib cage to allow for examination of the internal organs. The genitourinary tract, including the bladder (minus urine), prostate, and seminal vesicles, was excised and weighed. Lymph nodes surrounding the aortic bifurcation were harvested and analyzed for enlargement due to metastatic spread. Next, intestines, kidneys, liver, spleen, and lungs were removed and examined for overt metastases. At a minimum, samples of the prostate, seminal vesicles and periurethral lymph nodes were fixed in formalin overnight, paraffin embedded, sectioned at 5 μm, and stained with H&E. All histologic samples were blinded and evaluated by a certified genitourinary pathologist (K.J.W.).

Statistical analysis of prostate histology and lymph node metastases were done with Fisher’s exact tests comparing 5-aza-treated and control groups. Differences in genitourinary weight between the treatment groups were tested with the Wilcoxon rank test with Bonferroni adjustments being used to correct for the multiple comparisons. Kaplan-Meier methods were used for survival analysis between the PBS- and 5-aza-treated groups, and statistical significance was tested using the log-rank test. All analyses were done using SAS software version 9.12 (SAS Institute, Cary, NC) with P < 0.05 considered significant.

Results

Viral large Tag activates Dnmt1. As a model of pRb pathway inactivation, we used infection of primary human prostate epithelial cells with human polyomavirus BKV. This virus encodes six genes, including large (Tag) and small (tAg) T antigens, similar to those of the well-characterized SV40 polyomavirus (24). Polyomavirus Tag is capable of transforming many cell types through its inhibitory interactions with the pRb pocket protein family and p53 tumor suppressors (25). Transient infection of human prostate epithelial cells with BK virus led to a 6.8-fold
transcriptional activation of the DNMT1 gene by 48 hours after infection and 24-fold by 5 days after infection (Fig. 1A). Simultaneously, DNMT1 protein levels were up-regulated 6-fold 5 days after infection (Fig. 1B). In accordance with our previous findings showing E2F-mediated activation of DNMT1 (19), BK virus–mediated DNMT1 activation correlated with up-regulation of E2F1 and two well-characterized E2F target genes, cyclin E2 and PCNA (Fig. 1A and B). Additionally, stable immortalization of prostate epithelial cells with the SV40 TAg promoted sustained elevation of DNMT1 mRNA and protein (Fig. 1C and D). This stable DNMT1 activation again correlated with constitutive E2F target gene activation as shown by E2F1, cyclin E2, and PCNA levels (Fig. 1C and D). These data suggest that polyomavirus infection and TAg oncogene expression are capable of elevating DNMT1 levels, which may play a role in transformation.

**DNMT1 is overexpressed during TRAMP tumorigenesis.** Considering the regulation of DNMT1 by polyomavirus TAg, we examined Dnmt1 expression during prostatic tumorigenesis in the TRAMP mouse model. The TRAMP mouse model potently expresses the SV40 early region, including TAg, within the prostate (Fig. 2A; ref. 20). Although Dnmt1 RNA and protein levels were nearly undetectable in wild-type age-matched littermate control mouse prostate tissue, levels were greatly elevated in prostatic intraepithelial neoplasia (PIN), poorly differentiated prostate cancer, and metastatic lesions (Fig. 2A and B). Dnmt1 expression in these samples correlated well with increased E2F expression and activation of E2F target genes, such as thymidylate synthase (Tyms) and Pcna (Fig. 2A and B). The aberrant overexpression of Dnmt1 in TRAMP lesions suggests that altered DNA methyltransferase activity might play a role in the development of prostate cancer in TRAMP mice.

**5-Aza treatment prevents prostate enlargement and tumor progression.** To determine if DNA methyltransferase activity was required for TRAMP tumorigenesis, we treated mice with the DNA methyltransferase inhibitor 5-aza. The maximum tolerable dose of 5-aza was determined by treating mice with a range of doses (0.25–2.5 mg/kg) at varying frequencies (two or three times weekly). Mice were injected i.p. beginning at 6 weeks of age coinciding with the onset of transgene expression and continuing until 24 weeks of age (Fig. 3A). Treatments were ceased at 24 weeks as the majority of untreated TRAMP mice have significant dysplasia and/or neoplasia by this age (20, 26). Treatment groups receiving ≥0.5 mg 5-aza/kg of body weight given thrice weekly failed to reproducibly survive beyond 20 weeks of age, whereas the 0.25 mg 5-aza/kg body weight group treated twice weekly survived beyond 24 weeks of age without any detectable side effects (Supplementary Table S1).

When compared with wild-type C57BL/6 mice, PBS-treated TRAMP mice developed enlarged prostates and occasionally enlarged seminal vesicles by 24 weeks of age (wild-type PBS

---

**Figure 1.** Polyomavirus TAg stimulates DNMT1 expression in human primary prostate epithelial cells. A, RNA isolated from human prostate epithelial cells (hPrEC)–infected with BK virus for 0, 2, or 5 days was used for semiquantitative RT-PCR analysis of DNMT1, cyclin E2, E2F1, and hypoxanthine phosphoribosyltransferase 1 (HPRT) mRNA levels. B, whole-cell extracts were prepared from human prostate epithelial cells 5 days after mock or BKV infection and analyzed by SDS-PAGE and Western blotting with antibodies specific to DNMT1, PCNA, TAg, or actin. Semiquantitative RT-PCR (C) and Western blot (D) analysis were similarly done with lysates prepared from primary human prostate epithelial cells and human prostate epithelial cells immortalized with the SV40 TAg (hPrEC-TAg).

**Figure 2.** Dnmt1 is overexpressed in premalignant and malignant prostate cancer lesions in the TRAMP model. A, RNA lysates prepared from normal age-matched wild-type littermate control prostates or TRAMP prostates exhibiting prostatic intraepithelial neoplasia (PIN) or poorly differentiated prostate cancer were analyzed by semiquantitative RT-PCR with primers specific for Dnmt1, Tyms, E2F1, or hypoxanthine phosphoribosyltransferase (Hprt). B, protein lysates from normal age-matched wild-type prostates, TRAMP PIN lesions, TRAMP poorly differentiated prostate cancers (Cancer), or TRAMP periarcatic lymph node metastases (LN Mets) were analyzed by Western blot with antibodies specific to Dnmt1, Pcna, TAg, and actin.
24 versus TAg PBS 24; Fig. 3B). Genitourinary tract mass was considerably increased and varied widely (average, 2.39 g; range, 0.65-8.48 g) in PBS-treated TRAMP mice compared with similarly treated wild-type mice (average, 0.48 g; range, 0.38-0.58 g; Fig. 3B-D). However, when TRAMP mice were treated with 5-aza for 18 weeks, genitourinary tract mass stabilized quite reliably at a much smaller size (average, 0.67 g; range, 0.52-0.80 g; Fig. 3B and E). Although TRAMP genitourinary weights exhibited a statistically significant ($P = 0.0005$) decrease in weight with 5-aza treatment, a significant difference was not observed with 5-aza in wild-type mice (PBS average, 0.48 g; 5-aza average, 0.45 g). Although TRAMP genitourinary enlargement was much less pronounced at 16 weeks of age, 5-aza was still capable of diminishing this early increase in genitourinary weight (Fig. 3B). Therefore, it seems that the genitourinary enlargement observed in TRAMP mice is dependent upon DNA methyltransferase activity and can be inhibited by long-term 5-aza treatment.

Histologic examination of these tissues revealed that a majority (7 of 13, 54%) of 24-week-old PBS-treated TRAMP mice developed poorly differentiated prostate cancer, with a smaller fraction developing PIN lesions (6 of 13, 46%; Table 1; Fig. 4B and C). In stark contrast, mice treated with 5-aza developed exclusively PIN lesions (13 of 13, 100%) with no mice progressing to any stage of malignant disease (Table 1; Fig. 4D). TRAMP mice frequently exhibit metastases to the lymph nodes surrounding the aortic bifurcation posterior to the prostate. Consistent with prior reports (26), control-treated TRAMP mice developed lymph node metastases in 62% (8 of 13) of cases (Table 1; Fig. 4E). However, at 24 weeks of age, no (0 of 13) periaortic lymph node metastases were detected in 5-aza-treated TRAMP mice (Table 1; Fig. 4F). Additionally, at 24 weeks of age, PBS-treated TRAMP mice were frequently (9 of 13, 69%) observed to develop a phyllodes tumor-like histology of the seminal vesicles, whereas only 4 of 14 (29%) 5-aza-treated mice developed this abnormality (data not shown). Interestingly, whereas PBS-treated TRAMP mice develop more advanced diseases at 24 weeks of age compared with 5-aza-treated TRAMP mice, there is no statistically significant difference between the treatment groups in the development of PIN lesions at 16 weeks of age (Table 1). Therefore, it seems that DNA methyltransferase activity is strictly required for progression to malignant disease in the TRAMP model but is not required for the initiation of premalignant PIN lesions.

5-Aza increases survival in TRAMP mice. To determine if the prevention of tumorigenesis and metastasis observed in TRAMP mice treated with 5-aza led to an increased survival rate, groups of mice were monitored until they exhibited signs of marked morbidity or died spontaneously. PBS-treated TRAMP mice began
dying at 22 weeks of age, and 100% of the mice were dead by 43.57 weeks of age (Fig. 5). However, when TRAMP mice were treated with 5-aza the first death was observed at 31.71 weeks of age, and 84% (12 of 13) of these mice were still alive at 43.57 weeks when all control-treated TRAMP mice had died (Fig. 5). Interestingly, the increased survival of 5-aza-treated TRAMP mice is approximately equal to the 18-week treatment duration. These data suggest that 5-aza treatment is capable not only of preventing tumorigenesis and metastasis in TRAMP mice but also of dramatically increasing survival of these animals.

5-Aza does not affect transgene expression or cell proliferation. To rule out mechanisms of 5-aza action, which might be independent of its effect on DNA methyltransferase activity, we assessed transgene expression and S-phase markers. Use of the hormone-regulated probasin promoter for transgene expression could lead to misleading results if 5-aza affected hormone levels and therefore transgene expression. Transgene expression was assessed by Western blot analysis and immunohistochemistry in PBS- and 5-aza-treated TRAMP samples. Both techniques were unable to identify any difference in expression of the TAg transgene (Supplementary Fig. S1A and B).

Many chemotherapeutic anticancer agents elicit their effects through the induction of a relatively nonspecific growth arrest of rapidly dividing cells. The effect of 5-aza on cell cycle progression was assessed with the use of Pcna, a marker of proliferating cells in S phase. Again, both Western blot analysis and immunohistochemistry for Pcna showed no statistically significant difference in proliferation within PIN lesions following 5-aza treatment (Supplementary Fig. S1C and D). This finding is in agreement with previous data suggesting that 5-aza does not significantly block cell cycle progression (27).

5-Aza decreases hypermethylation observed in TRAMP prostates. To determine if the elevated Dnmt1 levels observed in TRAMP prostates correlated with hypermethylation and transcriptional silencing of any genes possessing CpG islands, we examined the expression and methylation status of the O6-methylguanine-DNA methyltransferase (Mgmt) gene. The protein product of the Mgmt gene plays an important role in the repair of mutagenic adducts from O6-guanine in DNA (reviewed in ref. 28). The 5′ region of the human and mouse MGMT genes have well-characterized CpG islands that are frequently hypermethylated and transcriptionally silenced in many cancers (29, 30). A semiquantitative RT-PCR analysis of Mgmt transcript levels in wild-type and TRAMP prostates revealed a considerable decrease in Mgmt expression in TRAMP prostates at 24 weeks of age (Fig. 6A). This transcriptional repression of Mgmt correlated with considerably elevated Dnmt1 levels (data not shown). To determine if this transcriptional repression was due to hypermethylation, the Mgmt CpG island

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
<th>Normal</th>
<th>PIN</th>
<th>WD</th>
<th>MD</th>
<th>PD</th>
<th>P value</th>
<th>LN Mets (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAg PBS 16</td>
<td>11</td>
<td>18</td>
<td>82</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>TAg Aza 16</td>
<td>11</td>
<td>36</td>
<td>64</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>NS (0.64)*</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>TAg PBS 24</td>
<td>13</td>
<td>0</td>
<td>46</td>
<td>0</td>
<td>0</td>
<td>54</td>
<td></td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>TAg Aza 24</td>
<td>13</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.005†</td>
<td>0</td>
<td>0.001†</td>
</tr>
</tbody>
</table>

Abbreviations: PIN, prostatic intraepithelial neoplasia; WD, well-differentiated prostate cancer; MD, moderately differentiated prostate cancer; PD, poorly differentiated prostate cancer; NS, not significant; NA, not applicable; LN, lymph node; Mets, metastasis.

*Fisher’s exact test comparing the frequency of PIN formation at 16 weeks of age between PBS and 5-aza groups.
†Fisher’s exact test comparing the frequency of cancer development at 24 weeks of age between PBS and 5-aza groups.

Figure 4. 5-Aza treatment prevents the development of prostate cancer in TRAMP mice. Representative H&E staining of prostates isolated from (A) a 24-week-old PBS-treated wild-type mouse displaying normal histology, (B) a 24-week-old PBS-treated TRAMP mouse displaying (B) PIN or (C) poorly differentiated prostate cancer, and (D) a 24-week-old 5-aza-treated TRAMP mouse displaying PIN lesions only. E, a periaortic lymph node from a PBS-treated 24-week-old TRAMP mouse displays prostate cancer metastasis, whereas (F) a similar lymph node from a 5-aza treated TRAMP mouse is normal. LN, normal lymph node. T, metastatic tumor. Bar, 100 μm.
(Fig. 6B) was analyzed by bisulfite DNA sequencing. DNA isolated from the prostates of three individual wild-type mice revealed a very low level of basal DNA methylation within this locus (Fig. 6C). However, prostates isolated from five separate control-treated TRAMP mice exhibited marked hypermethylation within discrete regions of the Mgmt CpG island (Fig. 6C). These results are consistent with similar patterns of methylation observed to induce heterochromatin and gene silencing of the human MGMT gene (31). When DNA was analyzed from multiple prostates of 5-aza-treated TRAMP mice, a considerable decrease in the extent of hypermethylation was observed compared with PBS-treated TRAMP mice (Fig. 6C). This decreased methylation in 5-aza-treated TRAMP mice correlated with a lack of transcriptional repression of Mgmt (Fig. 6A). These data show that elevated Dnmt1 levels correlate with hypermethylation of an important DNA repair component, and that 5-aza is capable of significantly preventing this hypermethylation-mediated silencing.

Discussion

Epigenetic modifications, such as DNA methylation, histone acetylation/deacetylation, and histone methylation, are currently believed to play a major role in human cancer. Understanding the genesis of these aberrant modifications in tumorigenesis will provide better insight into the use of DNA methyltransferase and histone deacetylase inhibitors in cancer treatment. In this study, we show that DNA methyltransferase activity is strictly required for progression from a premalignant state to advanced malignant disease in a mouse model of prostate cancer. During TAg-driven tumorigenesis, DNA methyltransferase inhibition with 5-aza is capable of effectively blocking tumorigenic transformation and DNA hypermethylation. These findings verify that DNA methyltransferase activity and DNA hypermethylation are critical for the initiation and progression of malignant disease.

Inactivation of the pRb pathway is considered an early event in many cancers (32). As we have shown previously, Dnmt1 is an E2F target gene that is aberrantly overexpressed in the absence of pRb (19). Thus, by activating the Dnmt1 promoter, early inactivation of the pRb pathway may provide an environment in which growth inhibitory and/or proapoptotic regulators are more readily silenced as a result of elevated DNA methyltransferase activity. Consistent with this hypothesis, exogenous overexpression of Dnmt1 in human fibroblasts has been shown to promote the hypermethylation of multiple tumor suppressor genes, including hypermethylated in cancer 1 (HIC1), estrogen receptor 1 (ESR1), and E-cadherin (CDH1; ref. 11). Dnmt1 overexpression also induced a transformed spindle cell morphology in rat fibroblasts (12) and supported growth of mouse 3T3 cells in soft agar (33). As predicted from in vitro observations, gastric cancer patients overexpressing Dnmt1 exhibited poorer tumor differentiation and DNA hypermethylation of multiple CpG islands (34). Through this mechanism, pRb inactivation might encourage the hyperproliferation and apoptosis resistance typically associated with cancer cells.
Indeed, we show hypermethylation and gene silencing of the Mgmt DNA repair gene in TRAMP tumors, which exhibit aberrant overexpression of Dnmt1. Loss of Mgmt expression may have significant implications for TRAMP tumorigensis, considering the role of Mgmt in the repair of mutagenic lesions following alkylation of DNA. Importantly, O4-methylguanine is capable of mispairing with adenine or thymine during DNA replication leading to GC-to-AT transition mutations (28). Mgmt binds to DNA via a helix-turn-helix motif and uses an arginine finger to rotate the alkylated base into the enzymes active site where the methyl group is transferred to Mgmt (28). Although high levels of exogenous alkylating agents may be toxic to cells lacking Mgmt, low level exposure to endogenous alkylators may promote tumorigenesis by permitting sister chromatid exchanges, chromosome aberrations, and recombination in addition to mutations. Results of a study using Mgmt-deficient cells suggested that a sublethal dose of an alkylator might create up to 900 mutations and 180 recombinations (35). Fortunately, treatment of TRAMP mice in a chemopreventive manner with 5-aza was capable of preventing the hypermethylation and silencing of Mgmt. Therefore, 5-aza treatment may maintain the expression of other critical tumor suppressor genes, which play key roles in preventing tumor progression in this mouse model of prostate cancer.

Interestingly, in addition to the classic inactivating mutations associated with the pRB pathway (i.e., cyclin D1 overexpression, CDK4 amplification, p16 methylation, pRb mutation and loss of heterozygosity, etc.), recent data has suggested a potential role for viruses in the etiology of prostate cancer. The human polyomavirus BKV genome was shown to reside within many sites of the urinary tract, including the kidneys, ureters, bladder, prostate, and urethra (36). More recently, a second study further showed the presence and expression of human polyomavirus BKV in 15 of 16 (94%) and 15 of 21 (71%) prostate cancers, respectively, through PCR and in situ hybridization (37). Immunohistochemistry also detected TAg protein within premalignant focal atrophic lesions and hyperproliferative inflammatory atrophy in 9 of 21 (43%) prostate cancer samples (37). These studies support the use of the TRAMP model in our study as the causative tumorigenic agent in these mice is the early region of the SV40 polyomavirus, including TAg.

Although we have shown 5-aza to be efficacious as a chemopreventive agent against prostate cancer in the TRAMP model, the drug’s utility in the clinical setting has thus far been limited. The drug is highly unstable and has a remarkably short plasma half-life of 12 minutes due to metabolic conversion by liver cytosine deaminases (38). This relative instability of the drug might explain why 5-aza has shown efficacy for leukemias and lymphomas (38) but has thus far had little effect on solid tumors, which require increased time for the drug to penetrate deep within tumor tissues (39). However, even in nonsolid tumors, treatment regimens have required multiple consecutive administrations of the drug via i.v. infusion (40). Additionally, 5-aza is toxic at higher doses in mice and humans causing myelosuppression, nausea, and vomiting (39). In our study, at a lower dose, we observed no overt side effects of drug treatment besides hypospermatogenesis and occasional germinal cell aplasia where spermatid precursor cells were lost, but Leydig and Sertoli cells were retained. For these reasons and the potential to induce global hypomethylation, which is also associated with cancer susceptibility and chromosomal instability (41, 42), this drug may not work well as a chemopreventive agent.

Despite these apparent limitations for 5-aza, there are alternatives being studied, which might have similar chemopreventive effects without significant toxicity. Zebularine, a cytidine analogue containing a 2-[1H]pyrimidinone ring, also inhibits DNA methyltransferase; however, in contrast to 5-aza, it is very stable with a half-life of 44 hours at 37°C and pH 1.0 and is minimally toxic (43). Although nontoxic, zebularine has thus far unfortunately required up to 1 g/kg body weight doses in mouse models (44), but cooperative activity has been shown between 5-aza and zebularine, suggesting that combined treatment modalities might improve drug performance (45). Another drug capable of inhibiting DNA methylation is procainamide. This nonnucleoside drug is nontoxic, likely functioning through binding of GC-rich sequences, and is currently used as treatment for cardiac arrhythmias (46). Finally, a readily available chemopreventive DNA methyltransferase inhibitor might be (-)-epigallocatechin-3-galate (EGCG), the major polyphenol from green tea. Although its mechanism of action is not yet understood, EGCG is capable of inducing demethylation of silenced tumor suppressor genes in the human esophageal cancer KYSE 510 cell line (47). Thus, additional studies are necessary to investigate the potential of both 5-aza and its alternatives in the treatment of prostate cancer.

In our study, tumor prevention seems to have occurred during the duration when 5-aza was being administered. The extended survival observed in 5-aza-treated TRAMP mice was approximately equal to the 18-week treatment duration throughout the survival curve. Considering that 5-aza did not seem to prevent the hyperproliferation associated with premalignant PIN lesions, 5-aza nucleotides incorporated into genomic DNA during the treatment duration were likely diluted out upon cessation of treatment due to continued DNA replication. The fact that tumors developed when 5-aza was removed further supports the notion that 5-aza inhibits malignant tumor progression, without affecting premalignant PIN formation.

Recent high-throughput oligonucleotide microarray screens for genes methylated in prostate cancer showed that a high percentage of genes exhibited hypermethylation and that these aberrantly methylated genes were often associated with transcriptional repression (48, 49). Our finding that DNA methyltransferase activity plays a critical role in prostate tumorigenesis supports the hypothesis that elevated DNA methyltransferase activity is involved in the hypermethylation of tumor suppressor genes during tumor progression. Although our data showed the ability of 5-aza to prevent prostate cancer, future experiments are necessary to address the ability of 5-aza to regress or stabilize preexisting cancer lesions. Additionally, studies investigating the cooperation of 5-aza with other treatment modalities, such as cytotoxic chemotherapy, radiation, cancer vaccines, and hormonal manipulations (i.e., chemical castration with prostate cancer) may identify superior treatment regimens.

Acknowledgments


Grant support: NIH grants DK61488 (M.L. Day) and AI060584 (M.J. Imperiale) and NIH Cancer Biology Training Fellowship ST32 CA09676 (M.T. McCabe).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Martin Sanda (Beth Israel Deaconess Medical Center, Boston, MA) for providing us with founding TRAMP mice and the University of Michigan Histology Core for excellent histologic support.
References


Inhibition of DNA Methyltransferase Activity Prevents Tumorigenesis in a Mouse Model of Prostate Cancer

Michael T. McCabe, Jonathan A. Low, Stephanie Daignault, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/1/385

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2006/01/17/66.1.385.DC1

Cited articles
This article cites 48 articles, 24 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/1/385.full#ref-list-1

Citing articles
This article has been cited by 24 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/66/1/385.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/66/1/385.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.