Inhibition of DNA Methyltransferase Inhibits DNA Replication*

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Ectopic expression of DNA methyltransferase transforms vertebrate cells, and inhibition of DNA methyltransferase reverses the transformed phenotype by an unknown mechanism. We tested the hypothesis that the presence of an active DNA methyltransferase is required for DNA replication in human non-small cell lung carcinoma A549 cells. We show that the inhibition of DNA methyltransferase by two novel mechanisms negatively affects DNA synthesis and progression through the cell cycle. Competitive polymerase chain reaction of newly synthesized DNA shows decreased origin activity at three previously characterized origins of replication following DNA methyltransferase inhibition. We suggest that the requirement of an active DNA methyltransferase for the functioning of the replication machinery has evolved to coordinate DNA replication and inheritance of the DNA methylation pattern.

Aberrant patterns of DNA methylation are observed in many cancer cells, and these changes occur in parallel with hyperactivation of DNA methyltransferase (DNMT-1) (1, 2). DNMT-1 is induced by nodal cancer signaling pathways (3–6), and a number of studies demonstrate that the hyperactivation of DNA methyltransferase plays a causal role in oncogenesis. For example, the expression of DNMT-1 in the antisense orientation reverses the tumorigenicity of Y1 adrenal carcinoma cells both in culture and in syngeneic mice (7), and the intraperitoneal injection of DNMT1 antisense oligonucleotides into LAF/1 mice bearing tumors derived from the syngeneic tumor cell line Y1 inhibits tumor growth (8). In addition, the reduction of DNMT-1 caused by either 5-aza-2′-cytidine treatment or by the mutation of one allele of the DNMT1 gene reduces the frequency of the appearance of intestinal adenomas in Min mice bearing a mutation in the adenomatous polyposis coli gene (9).

The mechanism by which the over-expression of the DNMT-1 induces tumorigenesis remains unresolved. An attractive model is that the hyperactivation of DNMT-1 leads to the hypermethylation and inactivation of a large number of genes that suppress tumorigenesis (10), tumor invasion (11), and angiogenesis (12). An alternative hypothesis is that the DNMT-1 protein, through protein-protein interactions, is involved in controlling either the entry into the S-phase of the cell cycle or the activity of the origins of replication and thereby progression through the cell cycle (13, 14).

To investigate how the inhibition of DNMT-1 results in the inhibition of tumorigenesis, we have developed phosphorothioate-modified hemimethylated oligonucleotides that, in the presence of a lipophilic carrier, can enter into the nucleus of cancer cells in culture, form a stable complex with DNMT-1, and specifically inhibit its activity with an EC50 of approximately 60 nM (15, 16). We have also developed an inactive analog of this phosphorothioate hemimethylated inhibitor of the same sequence, which does not form a stable complex with DNMT-1 and does not inhibit its activity, that can serve as an experimental control (15, 16). In addition, antisense oligonucleotides and an adenovirus expressing DNMT-1 antisense mRNA were used to test the hypothesis that the inhibition of DNMT-1 directly affects the growth of A549 cells by inhibiting DNA replication.

MATERIALS AND METHODS

Oligonucleotide Treatment and Thymidine Incorporation—A549 non-small cell lung carcinoma cells (ATCC, CCL 185) were treated with the relevant oligonucleotide at 100 nM, which was mixed with 6.5 µl of Lipofectin (2 mg/ml; Life Technologies, Inc.) and 1 ml of OptiMEM serum-free medium as described previously (15). The dose of oligonucleotide was determined by preliminary dose-response assays to result in the maximum inhibitory activity with essentially no nonspecific toxicity (data not shown). The direct inhibitor used in our study is a phosphorothioate-modified hemimethylated hairpin of the sequence 5′- CTTGAA(methyl)CGGAT(methyl)CGTTTCGATCCGTTCAG-3′ (3118); the control oligonucleotide is identical and is also phosphorothioate-modified but has been modified at all the 2′-O-methyl positions of the sugar backbone (3098). Both oligonucleotides were tagged with fluorescein at their 5′ end. The antisense DNMT1 oligonucleotide used in our study and the mismatch control are phosphorothioate-modified: DNA MT1 antisense, 5′-AAGCATGAGACCTGTCC-3′; and mismatch control, 5′-AAGCATGAGACCTGTCC-3′. The oligonucleotide containing medium was removed from the cells and replaced with regular growth medium after 4 h. The treatment was repeated after 24 h. DNA synthesis was determined at the indicated time points after initiation of the first treatment by measuring [3H]thymidine incorporation into DNA following an 8-h pulse with 66 Ci/ml [3H]thymidine.

Adenoviral Infection—DNMT1 full-length cDNA was cloned into the AdEasy shuttle vector pAdTrack cytomegalovirus in the XbaI site in the antisense orientation. Adenoviral recombination and preparation of infectious particles in HEK 293 cells was performed as described previously (17). A549 cells were infected with either the control AdEasy virus or the AdEasy DNMT1 antisense at a multiplicity of infection of 50 or 150. 100% of the cells were infected as determined by visualizing green fluorescent protein under a fluorescence microscope. 48 h after transfection, the cells were pulsed with thymidine as described above, and nuclear extracts were prepared for determination of DNA methyltransferase activity (15).

Mitotic Index—Cells were treated twice with hairpin oligonucleotides at 24-h intervals. 48 h after the start of the first treatment, the cells were treated with 1 µg/ml colcemid (Life Technologies, Inc.). At the times indicated, the cells were fixed with −20 °C methanol, stained

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‡ The abbreviations used are: DNMT-1, DNA methyltransferase; BrdUrd, bromodeoxyuridine; kb, kilobase pair; PCR, polymerase chain reaction; DAPI, 4,6-diamidino-2-phenylindole; PCNA, proliferator cell nuclear antigen; PBS, phosphate-buffered saline.
with 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI; ICN Biomedicals), mounted, and examined.

Isolation of Newly Synthesized DNA—A549 cells were treated twice with oligonucleotide DNA methyltransferase inhibitors at 24-hour intervals as described above. The newly synthesized DNA was isolated from equal amounts of total DNA by immunoprecipitation with an anti-BrdUrd antibody as described previously (18) followed by the gel isolation of strands 0.4–1.2 kb in size. To verify our results, a second recently described method of enriching for nascent DNA, by selecting for 5'-RNA-DNA chains from early replication bubbles, was used (19). Equal amounts of total DNA extracted from the cells was treated with exonuclease, as described previously, to eliminate all of the nicked 5'-phosphorylated DNA, leaving intact nascent DNA that has an RNA primer at its 5' position. The nascent DNA-enriched samples were subjected to competitive PCR to quantify the amount of nascent DNA initiated from each origin.

Competitive PCR—Competitive PCR was performed as described previously, using the previously described primers and competitors for the β-globin, c-myc, and dnm1 origins of replication (20–22).

Hydroxyurea Treatment—Cells were serum-starved in OptiMEM for 24 h. The medium was then replaced with serum-free OptiMEM containing 800 µM hydroxyurea and incubated for an additional 24 h. To release the cells from the G1/S block, the cells were washed twice with warm PBS and then grown in Dulbecco's modified Eagle's medium (low glucose) supplemented with 10% fetal calf serum and 2 mM glutamine. Oligonucleotide treatment was performed during the last 4 h of the serum starvation and immediately prior to treatment with hydroxyurea as described above.

RESULTS

Inhibitors of DNMT-1 Slow Cell Growth, the Progression through the Cell Cycle, and the Rate of DNA Replication—We have previously demonstrated that the treatment of A549 cells with direct inhibitors of DNMT-1 results in an inhibition of their anchorage-independent growth (15). In Fig. 1A we show that the fluorescein-tagged inhibitor (3118) inhibits DNMT-1 activity from A549 cells in a dose-dependent manner relative to the inactive analog (35% inhibition at concentration of 50 nM and 65% inhibition at a concentration of 100 nM) as determined by an in vitro DNMT-1 assay (15).

To determine whether the DNMT-1 inhibitor inhibits DNA replication, we assayed the rate of [3H]thymidine incorporation following either single or double treatments. The results, shown in Fig. 1B, demonstrate that the direct inhibitor of DNMT-1 causes a 50% inhibition in DNA synthesis 24 h after initiation of treatment relative to the inactive analog (30 versus 80%, respectively). This level of inhibition of DNA synthesis remains similar 24 or 72 h after a second oligonucleotide treatment (which corresponds to 48 and 96 h after the start of the experiment).

To verify that the inhibition of DNA replication by 3118 is a consequence of inhibition of DNMT-1 activity and not a different cellular response triggered by 3118, we inhibited DNA methyltransferase by expressing DNMT1 antisense mRNA. A549 cells were infected with either an AdEasy adenovirus expressing the DNMT1 cDNA in the antisense orientation or a control AdEasy virus expressing green fluorescent protein as a methyl donor as described previously (29). The results presented are an average of three determinations ± S.D. (the total count obtained for untreated cells was ~35,000 dpm). C, A549 cells were infected with either AdEasy DNMT1 antisense (a-Metase) or AdEasy control (GFP, green fluorescent protein) at a multiplicity of infection (MOI) of 50 or 150. 48 h later, 3 µg of nuclear extracts prepared from the control and infected cells were assayed for DNA methyltransferase activity as described previously (29). The results presented are an average of three determinations ± S.D. D, reduction in [3H]thymidine incorporation by AdEasy DNMT1 antisense. The bars represent the percent incorporation of [3H]thymidine over an 8-h incubation period of cells treated with the direct inhibitor (3118) and cells treated with the inactive analog (3188) relative to cells treated with Lipofectin only. Triplicate determinations of each time point were made, and the results shown are the mean of two independent experiments ± S.D. The maximal mitotic index of the cells treated with the direct inhibitor was 24% and was achieved 26 h after the start of the colcemid treatment. The maximal mitotic index of the cells treated with the inactive analog was 37% and was achieved 26 h after the start of the colcemid treatment (Fig. 2D). These results demonstrate that a direct inhibitor of DNMT-1 slows the progression through the cell cycle.

Inhibition of DNMT-1 Inhibits Origin Activity—The rate of
DNA synthesis is normally dependent upon the number of active origins. To determine whether the inhibition of DNA methyltransferase results in an inhibition of origin activity and whether this effect is dependent on the state of methylation of origins of replication, competitive PCR was used to quantify the abundance of two well characterized origins, \(\beta\)-globin and \(c\)-myc (Fig. 3), in newly synthesized DNA as described previously (22). These origins are differentially methylated (22) and are thought to replicate at different points in the S-phase. A549 cells were treated with either the direct inhibitor (3118) or the control (3188) for 4 h and then pulsed with BrdUrd for 1 h. Newly synthesized DNA was prepared by immunoprecipitation of BrdUrd pulse-labeled DNA with anti-BrdUrd antibodies followed by the gel isolation of strands 0.4–1.2 kb in size. To standardize the experiment, because of differences in primers and competitor amplification efficiencies, competitive PCR of both \(\beta\)-globin and \(c\)-myc origins was performed using A549 genomic DNA (Fig. 3, A–D). The results (Fig. 3, E–G) show that the DNMT-1 inhibitor (3118) inhibits the activity of both \(\beta\)-globin and \(c\)-myc origins of replication to a similar extent, suggesting that inhibition of DNMT-1 inhibits the origins of replication irrespective of their state of methylation.

Inhibition of DNMT-1 Inhibits Initiation of DNA Replication—To further study how inhibition of DNMT-1 affects DNA replication, we used the DNA synthesis inhibitor hydroxyurea. Hydroxyurea, an inhibitor of ribonucleotide reductase, reduces the pool of deoxynucleotides in the cell, resulting in the blocking of progression of pre-existing replication forks and late origins but not initiation at early firing origins (24). Therefore, any added effect of the inhibitors would have to be achieved by a mechanism that is independent of the mechanisms affected by hydroxyurea. A549 cells were treated with Lipofectin carrier alone, the direct inhibitor (3118), or the inactive analog (3188) for 4 h, followed by a 24-h treatment with 800 \(\mu\)M hydroxyurea (MO, MF 1–3). The cells were then washed twice with PBS and incubated in complete medium for 3 h (MO, MF 4–6). The rate of initiation of the \(c\)-myc origin of replication (MO1–6) and of a secondary initiation site located 7 kb downstream (MF1–6) was determined by competitive PCR of RNA-primed DNA that was resistant to \(\lambda\)-exonuclease as described previously (19). Nascent DNA differs from genomic DNA by being RNA primed. Fig. 4A shows that the \(\lambda\)-exonuclease treatment eliminates effectively all the genomic DNA and the dephosphorylated plasmid DNA control. As shown in Fig. 4B and quantified in Fig. 4, C and D, hydroxyurea treatment alone does not inhibit the firing of the \(c\)-myc origin of replication (MO1 and MF1), as expected. Fluorescence-activated cell sorter analysis demonstrated that the treatment has indeed arrested all of the cells at early S-phase as expected (data not shown). If the direct inhibitor affects the elongation of nascent DNA strands rather than initiation, then the results (MO2 and MF2) should be the same as treatment with hydroxyurea alone (MO1 and MF1). However, as observed in Fig. 4B and quantified in Fig. 4, C and D, the rate of initiation from the \(c\)-myc origin of replication is significantly inhibited by the direct inhibitor (MO2 and MF2) but not by the inactive analog (MO3 and MF3). To test whether the effect of the DNMT-1 inhibitor is reversible or whether it has a general toxic effect on the cell, we measured the nascent DNA abundance following release from the hydroxyurea block and growth in regular medium for 3 h (Fig. 4, A–D).
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**Fig. 4.** Two different DNMT-1 inhibitors in hydroxyurea-treated cells inhibit initiation from the c-myc origin of replication. A549 cells were treated with 800 μM hydroxyurea alone (MO1 and MF1), hydroxyurea and the direct inhibitor 3118 (MO2 and MF2), or hydroxyurea and the inactive analog 3188 (MO3 and MF3) for 24 h. The cells were then washed twice with PBS and incubated in complete medium for 3 h (MO4–6 and MF4–6 correspond to washed MO1–3 and MF1–3, respectively). Nascent RNA-primed DNA was prepared by digesting equal amounts of genomic DNA with λ-exonuclease as described previously (19). A, A549 genomic DNA isolated from cells treated with hydroxyurea alone, hydroxyurea and the antisense oligonucleotide, or hydroxyurea and the mismatch control oligonucleotide was phosphorylated and treated with either λ-exonuclease (λ-EXO) (+) or the incubation buffer alone (−) in the presence of dephosphorylated plasmid DNA (pDNA) to control for both phosphorylation and full digestion. The samples were fractionated on a 1% agarose gel and stained with EtBr. B, a competitive PCR assay was used to measure nascent DNA abundance. To normalize the differences in primer and competitor amplification efficiencies, A549 genomic DNA (from A549 cells) as a control for variability in primer and competitor amplification efficiency, nascent DNA versus target DNA was determined by densitometry and plotted as described in Fig. 3, C and D (data not shown). The calculated percentage of nascent DNA versus control at the c-myc origin (C) and a sequence ~7 kb away (D) were then plotted as bar graphs. Filled bars represent samples treated with hydroxyurea alone, empty bars represent samples treated with hydroxyurea plus the direct inhibitor (3118), and shaded bars represent samples treated with hydroxyurea plus the inactive analog (3188). The lines under the plot indicate whether the samples were washed following hydroxyurea treatment (HU WASH) or not (HU B–D). As shown in Fig. 4B and quantified in Fig. 4, C and D, none of the cells (i.e. those untreated (MO4, MF4), the cells treated with the direct inhibitor (MO5, MF5), or cells treated with the inactive analog (MO6, MF6)) had any substantial inhibition of replication from the c-myc origin. These results (MO4–6 and MF4–6) demonstrate that the inhibitory effects observed on DNA replication by the DNMT-1 inhibitor are reversible and thus are not toxic.

The data presented in Figs. 3 and 4 shows that inhibition of DNMT-1 dramatically reduces the abundance of nascent strands near origins. However, this inhibition has a significantly less pronounced effect on overall DNA synthesis as measured by incorporation of [3H]thymidine (Fig. 1). The discrepancy between the extent of inhibition of nascent strand abundance near origins and the extent of inhibition of [3H]thymidine incorporation can most simply be explained by the hypothesis that inhibition of DNMT-1 leads to inhibition of initiation and not to inhibition of ongoing replication fork movement.

To ascertain that the inhibition of origin activity observed with 3118 is a consequence of DNA methyltransferase inhibition, we measured origin activity following inhibition of DNA methyltransferase by a previously characterized DNMT1 antisense oligonucleotide (25). A549 cells were treated with Lipofectin carrier alone, the DNMT1 antisense oligonucleotide (MD88), or the mismatch control (MD208) for 4 h, which was followed by a 24-h treatment with 800 μM hydroxyurea. The antisense oligonucleotide (MD88) inhibits DNMT-1 activity from A549 cells relative to the mismatch control (50% inhibition at a concentration of 80 nM) as determined by an *in vitro* DNMT-1 assay (data not shown). The rate of initiation of the c-myc origin of replication and of two initiation sites located in the dnmt1 locus (Fig. 4) (20) was determined by competitive PCR of RNA-primed DNA that was resistant to λ-exonuclease. As observed in Fig. 4E and quantified in Fig. 4F, the origin activity from both the c-myc origin of replication and the two *dnmt1* initiation sites of replication is significantly inhibited by the DNMT1 antisense relative to the mismatch oligonucleotide.

**DISCUSSION.** Our experiments demonstrate that the inhibition of DNMT-1 inhibits the activity of at least 3 origins of replication. The mechanism through which inhibition of DNMT-1 leads to inhibition of DNA replication remains unsolved. A simple and attractive hypothesis is that inhibition of DNMT-1 leads to passive demethylation and activation of putative tumor suppressors when DNA is synthesized in the absence of DNMT-1 activity. It has been demonstrated that prolonged treatment with 5-aza-2’-deoxycytidine or an antisense inhibitor of DNMT-1 can lead to a sustained induction of p16 (25–27). However, in both cases, the immediate cytostatic effects on cell growth have been shown to be independent of the induction of p16 (25–27).

In A549 cells p16 is deleted, however, it is possible that the passive demethylation and activation of another putative tumor suppressor is involved. Two observations are inconsistent...
with this hypothesis. First, if the DNMT-1 inhibitor causes passive demethylation by depleting the DNMT-1 pool, demethylation of a specific locus should be a stochastic event. If this is true, then the level of demethylation of a specific locus should increase with successive rounds of replication. In contrast, the results presented in Fig. 1B show that the inhibition of DNA synthesis is both rapid and does not increase with time. Second, the direct inhibitor of DNMT-1 is effective in the presence of hydroxyurea, which inhibits DNA replication. Following the inhibition of DNMT-1, DNA methylation activity of DNMT-1 becomes targeted to sites of DNA replication does not proceed in the absence of DNA methylation. Synthesis of DNMT-1 is induced at the entry to the S-phase (29, 30), and it becomes targeted to sites of DNA replication (31, 32) where it interacts with PCNA (33). Consistent with its localization during S-phase, we and others have previously shown that DNA replication and methylation are concomitant events (22, 34). The repression of DNA replication following the inhibition of DNMT-1 might be an additional mechanism. However, the results shown in Fig. 3 are inconsistent with this hypothesis, since they demonstrate that both methylated and nonmethylated origins of replication are similarly affected.

Yet another hypothesis is that the direct inhibitor as well as antisense treatment disrupts protein-protein interactions between DNMT-1 and other proteins of the replication complex, such as the previously demonstrated interaction with PCNA (33), required for DNA replication. Interestingly, it has recently been reported that a protein related to DNMT-1 is expressed in Drosophila and associates with PCNA (35). Because Drosophila DNA does not bear methylated cytosines, this report supports the hypothesis that DNMT-1 might have additional functions in the replication fork. It is possible that the DNA methylation activity of DNMT-1 has evolved to coordinate the processes of DNA replication and inheritance of the DNA methylation pattern (14).

Additional experiments are required to establish the details of the mechanisms that are responsible for arresting DNA replication following the inhibition of DNMT-1. However, the inhibition of DNA replication by a direct inhibitor of DNMT-1, as well as an antisense oligonucleotide and an antisense adenoviral vector, strongly suggests that DNMT-1 activity is essential for the activity of origins of replication in at least some cancer cell lines.

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