DNA Methyltransferase Inhibition in Normal Human Fibroblasts Induces A p21 Dependent-cell Cycle Withdrawal

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Abbreviations: CdR, 5-aza-2-deoxycytidine; DNAMeTase, DNA methyltransferase; PD, population doublings; CMV, CMV-mj-hel-1; 041, MDAH041; hTERT, telomerase reverse transcriptase protein; SV40, simian virus 40.
SUMMARY

Maintenance of methylation patterns in the mammalian genome by DNA (cytosine-5) methyltransferases (DNAMeTase) is required for normal cell and tissue function. Inhibition of DNAMeTase in cultured cells induces the expression of p21, a cyclin dependent kinase (Cdk) inhibitor critical for cells to enter replicative senescence. We investigated the effects of DNAMeTase inhibition in normal human fibroblasts and found that it induces an irreversible growth arrest. Cells arrested by DNAMeTase inhibition became enlarged and had a flat morphology, exhibited an increased expression of collagenase and p21, and the DNA synthesis block could be overcome by the introduction of the SV40 large T antigen, all characteristics of senescent cells. In contrast, normal human fibroblasts lacking a functional p21 gene fail to undergo cell cycle arrest following DNAMeTase inhibition, indicating that p21 is an essential component of this arrest. Furthermore, DNAMeTase activity was reduced as cells approached the end of their proliferative potential. These data suggest that DNAMeTase could be an integral part of the mechanisms by which cells count the number of cell divisions completed and initiate a signaling cascade that ultimately results in the senescent phenotype.
INTRODUCTION

Most eukaryotic cells do not divide indefinitely. This phenomenon of limited proliferative capacity, termed replicative senescence, was initially documented in cultures of human fibroblasts (1). Since then, it has been extended to a number of cell types and species and has become a model for aging at the cellular level. Replicative senescence is characterized by an irreversible growth arrest that is the consequence of repeated cell division. The number of divisions that normal cells complete before they enter senescence is constant under standard conditions and depends on the species, cell type, age and genetic background of the donor (2). This suggests the existence of a mechanism by, which cells sense the number of divisions completed. Telomere erosion, resulting from the incapacity of DNA polymerase α to replicate the end of linear chromosomes, has emerged as a major candidate for the counting mechanism of human cells (3). The telomere hypothesis of cellular aging has been greatly strengthened by experiments demonstrating that ectopic expression of the catalytic subunit of telomerase (hTERT) before senescence, prevents telomere shortening and allows indefinite cell proliferation (4). The expression of hTERT correlates with the presence of telomerase activity in human cells, and the role of telomerase in immortalization is generally believed to involve the prevention of telomere erosion. However, it was recently reported that telomerase extends the lifespan of SV40-transformed human fibroblasts without net telomere lengthening (5) and that stable clones expressing limiting amounts of hTERT and having shorter telomeres than the parental senescent cells continue to proliferate (6). This indicates that the resolution of the telomere replication problem is not always necessary for extension of life span. In addition, the chromosomal instability that occurs when telomeres erode sufficiently, causing a crisis like state in pre-immortalized SV40 transformed cells, is not a characteristic of the normal
aging phenotype and occurs only when senescence has been circumvented. Furthermore, immortal cell lines in which telomerase was inhibited by a dominant negative form of the enzyme undergo apoptosis instead of entering senescence (7,8).

The gradual loss of DNA methylation has been proposed as an alternative counting mechanism (9, 10). In human cells, 60-90% of the cytosines in CpG dinucleotides are methylated at the carbon 5 position, leaving a minor portion of the genome methylation free. Methylation of CpG islands is associated with transcriptional silencing and the formation of condensed chromatin structures enriched in hypoacetylated histones (11,12). DNA methylation has been shown to be essential for normal development, X chromosome inactivation, imprinting and has been suggested to play a role in in vitro cellular aging. The extent of CpG methylation decreases during serial passage of normal cells in culture (9, 13) and during aging of organisms (14, 15). On the other hand, most immortal cell lines maintain constant levels of genomic DNA methylation. However, it is not known whether the aging related loss of methylation involves specific regions of the genome or if it is a stochastic process.

The transfer of a methyl group from S-adenosylmethionine to the 5 position of cytosines in CpG dinucleotides is catalyzed by the enzyme DNA (5-cytosine) methyltransferase (DNAMeTase) (16). DNAMeTase activity has been shown to be elevated in cancer cells in vitro, in tumors in vivo (17) and in transformation triggered by oncogenic ras or SV40 T antigen (18, 19). In addition, increased DNAMeTase levels are required to maintain the phenotype of fibroblasts transformed with ras and with the fos oncogene (20), suggesting that the maintenance of DNAMeTase activity and hence DNA methylation levels, is an important step in cellular transformation. Consistent with the loss of DNA methylation, DNAMeTase activity was observed to decrease with increasing population doublings in serially passaged normal fibroblasts (21).
One of the major effectors of the senescence phenotype is p21. p21 mRNA and protein are significantly increased as fibroblasts enter senescence (22). Human diploid fibroblasts in which both copies of the gene have been inactivated by homologous recombination fail to undergo normal senescence (23). They have an extended in vitro life span and rather than attaining a senescent state they enter a crisis like state in which DNA synthesis and cell death occur simultaneously. In addition, p21 appears to be needed for the induction of the senescent like state by many treatments such as sodium butyrate, which induce premature growth arrest and many features of senescence (24). Although it is known that p21 blocks the action of the cyclin dependent kinases (25) and prevents the phosphorylation of Rb (26) thereby preventing the induction of many of the genes needed to traverse the G1/S boundary (27), the role of p21 in cellular senescence is not completely understood and a mechanism for the induction of p21 at the end of the in vitro life span of a fibroblast cell culture has not yet been convincingly demonstrated. Very recently it was reported that p21 could be induced by inhibition of DNAMeTase (28, 29). Thus, it appears that DNAMeTase itself, independent of DNA methylation, can inhibit gene transcription. The exact mechanism of inhibition is not known, but DNAMeTase does have a repressive domain and it interacts with repressive complexes that include HDACs, which appears to contribute to a repressive effect on the p21 promoter (30, 31, 32). Since DNAMeTase activity decreases during cellular aging, the repressive effect on the p21 promoter may be overcome and chromatin structure surrounding the p21 promoter may be more active resulting in increased p21 transcription and cellular senescence.

In this report we describe the effect of DNAMeTase inhibition on normal human diploid fibroblasts. We demonstrate that inhibition of DNAMeTase induces a senescence-like cell cycle arrest that is mediated by and requires functional p21. Our results support the hypothesis that
DNAMetase activity and/or methylation levels could act as a cell division counting mechanism in human cells.

**EXPERIMENTAL PROCEDURES**

**Cell culture.** HCA2, human diploid fibroblasts isolated in this laboratory from neonatal foreskin and all human cells lines used were maintained and made quiescent as described previously (33). Human lung fibroblasts (LF1) and LF1 derivatives with a homozygous deletion of both p21 alleles designated H07.2-1, were a generous gift from J. Sedivy (23).

**Determination of the 5mC content.** Genomic DNA was isolated using the DNAZol DNA extraction kit (Molecular Research center, Inc.) according to manufacturer’s directions. RNA was removed by alkaline hydrolysis (0.5 M NaOH, 37°C for 1h). The 5mC content in DNA samples was determined by HPLC analysis of enzymatic hydrolysates of DNA. 10 µg of DNA was digested at 37°C for 3 hours using 2 units of microccocal nuclease (USB, Ohio) and 2µg of spleen phosphodiesterase II (Roche Diagnostics Corp, In) in 10 mM CaCl₂ and 20 mM sodium succinate, pH 6.0. The resulting 3’ deoxymonophosphate nucleosides were further hydrolysed by overnight incubation at 37°C with 20 units of alkaline phosphatase (Amersham Pharmacia Biotech). Samples were injected into a Beckman Ultrasphere ODS, 4.6 mm X 25 cm (5µm particle size) column at room temperature, programmed as follows: 100% buffer A (2% methanol in 0.05M potassium phosphate, pH 4.5) for 10 minutes, injection of the samples, elution for 20 min in 100% buffer A, followed by buffer B (9% methanol in 0.05M potassium phosphate, pH 4.5) over a 5 min linear gradient up to 100% buffer B for 10 min. HPLC mobile phase was delivered to the column at 0.3 ml/min. Absorbance at 280 nm was recorded. The percent methylcytosine in the genome was
determined as a ratio of the area of the 5mC peak to the total area of methylcytosine and cytosine residues in the sample.

**CdR treatment.** Cells were plated at a density of $10^4$ cells/cm$^2$ and 24 hr later treated with 0.5 µM CdR (Sigma Chemical). The medium was changed every 24 hr for medium containing fresh CdR for the time required to control cells to achieve 3 population doublings.

**Determination of cell cycle profile.** Cells ($2\times10^6$) were fixed with 70% ethanol for 30 min, stained with 50 µg/ml propidium iodide and treated with 10 µg/ml RNase A. DNA content at each cell cycle stage was determined by flow cytometry.

**DNA methyltransferase activity.** Cells were harvested by trypsinization, washed twice in PBS, and resuspended in 0.1 ml hypotonic lysis buffer containing 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM DTT, 0.01% NaN$_3$, 10% glycerol, 1% Tween 80, 60 µg/ml phenyl-methanesulfonyl fluoride (PMSF), and 100 µg/ml RNase A. The cells were lysed by four cycles of freezing in dry ice-ethanol and thawing at 37°C. Protein concentration of the supernatant, after centrifugation of the cell lysates at 15,000Xg for 20 min, was determined by the Bradford method (BioRad, Richmond CA). The enzyme activity was measured as previously described (16), with slight modifications. Briefly, a 20 µl reaction mixture containing 5 µg of cell extract protein, 0.5 µg of a hemimethylated oligonucleotide duplex corresponding to the imprinted locus *SNRPN* (small nuclear riboprotein-associated peptide N) exon-1, and 3.3 µCi S-adenosyl[methyl-3 H]methionine (92 Ci/mmoll; Amersham Pharmacia Biotech). The mixture was incubated at 37°C for 2 hr, and the reaction terminated by the addition of 300 µl of a solution containing 1% sodium dodecyl sulfate (SDS), 2 mM EDTA, 3% 4-aminosalicylate, 5% butanol, 125 mM NaCl, 0.25 mg/ml salmon sperm DNA, and 1 mg/ml proteinase K. After incubating for a further 30 min at 37°C, the reaction mixture was
extracted with an equal volume of phenol/chloroform/isoamyl alcohol and ethanol precipitated. DNA was dissolved in 0.3 N NaOH and incubated at 37°C for 2 hr. DNA was collected on a glass fiber filter disc, saturated with 1 mM nonlabeled AdoMet, and washed with 5% TCA followed by 70% ethanol. The filter was air-dried, and the radioactivity measured by β scintillation counting in 5 ml of scintillation fluid. After subtracting background, the radioactivity incorporated into the DNA as a measure of DNAMeTase activity was determined. Reactions were performed in triplicate and the results expressed as the mean +/- SD.

**Oligonucleotides.** 2'-O-methylphosphorothioate oligonucleotides were used in the antisense experiments: DNAMeTase antisense (MG88): AAGCATGAGCACGTTCTCC and unrelated control oligonucleotide (Unr): ATACAAACATGACAATAGATCG (bold nucleotides are 2'-O-methyl modified). The following oligonucleotides were used as substrate for the DNAMeTase activity determinations: The hemimethylated duplex was prepared by annealing equimolar amounts of SNRPN-meth:

CTTGCCMGCTCCATMGMGTCACTGACMGCTCCTCAGACAGATGMGTCAGGCATCTCM
GGMGGCMGCTCCACTCTG (methylated single strand oligonucleotide) and SNRPS-unmeth:

CTTGCCMGCTCCATMGMGTCACTGACMGCTCCTCAGACAGATGMGTCAGGCATCTCM
GGMGGCMGCTCCACTCTG (unmodified single strand oligonucleotide).

The complementary oligonucleotides were incubated in annealing buffer (50 mM Tris-HCl, pH 7.4, 1 mM Na₂EDTA, and 100 mM NaCl) at 95 °C for 5 min followed by 65 °C for 15 min and 37 °C for 15 min (34). The full-length duplexes were purified on a 15% polyacrylamide gel, dissolved in TE, and stored at 20 °C.
Oligonucleotide treatment. Cells ($5 \times 10^5$) were electroporated on 3 sequential days (300V, 960µFD) with 100 nM of the hybrid 2’-O-methylphosphorothioate DNAMEtase antisense or unrelated oligonucleotide in freshly made cytomix buffer (120 mM KCl, 0.15 mM CaCl$_2$, 10 mM K$_2$HPO$_4$, 10 mM KH$_2$PO$_4$, 25 mM Hepes, 2 mM EGTA, 5 mM MgCl$_2$, 2 mM ATP, 5 mM glutathione, pH 7.6). After each electroporation the cells were incubated in complete medium for 48 hr in the presence of 40 nM oligonucleotide.

Transient transfection of an antisense p21 expression vector. The CMV-AS-p21 plasmid contained 1-165 nucleotides of p21 cDNA in the opposite orientation. CMV control vector (empty vector) and CMV-AS-p21 were cotransfected with CMV-EGFP plasmid into HCA2 cells (PD 19) using Lipofectamine Plus (Gibco BRL). Transfection medium (0% FBS) was replaced with 0.5 µM CdR, 10% FBS medium 3 hr after plasmid delivery.

SA-β-galactosidase activity. Staining for SA- β-galactosidase (β−gal) activity was performed as described (35).

[^H] Thymidine labeling. Thymidine incorporation was performed as described previously (33).

SV40 T antigen electroporation. PSV7, a SV40 early promoter-driven T antigen expression vector (10 µg) was introduced into HCA2 cells by electroporation (300V, 960µFD) using 0.5X10$^6$ cells in 400 µl of freshly made cytomix buffer.

Determination of the colony size distribution. Cells were plated at a density of 20 cells per 60 mm dish and incubated undisturbed for two weeks in 10% FBS. Dishes were then fixed for 5 minutes in 1% glutaraldehyde, washed gently with water and stained with 1% crystal violet. The number of cells in the individual colonies was determined using a dissecting microscope.
**Northern blot analysis.** Northern analysis was performed according to standard procedures. ³²P-labeled DNA probes for human mRNAs were prepared by random priming.

**Western blot analysis.** Samples containing equal amounts of protein (20 µg) from cell lysates were separated by SDS-PAGE and then transferred to a nylon membrane. Proteins were detected by incubation with the indicated antibodies and enhanced chemiluminescence (ECL, Amersham corp.).

**RESULTS**

**Total 5-methylcytosine content in normal diploid fibroblasts is reduced during cellular aging.**

The normal human diploid fibroblast cell line HCA2 can replicate in culture for 80-90 population doublings (PD). In order to determine the rate of reduction in total DNA methylation during in vitro aging in HCA2 cells, we measured 5-methylcytosine (5mC) content in genomic DNA from cells at increasing PD. Reverse phase HPLC revealed that 5mC content was reduced from 5.3 % (+/- 1.03) to 2.2 % (+/- 0.61) from PD 27 to PD 80 in culture (figure 1A). The decrease in DNA methylation levels during in vitro aging was accompanied by a decrease in DNAMeTase activity (figure 1B). The tritiated thymidine labeling index of the above cultures indicated that the decrease in DNAMeTase activity was not related to the percentage of proliferating cells, but rather related to the proliferative capacity of the culture.

DNA methylation levels can be manipulated by inhibition of the DNA (cytosine) methyltransferase (DNAMeTase) (36). To induce a premature decrease in global DNA methylation levels we treated HCA2 cells with 5-aza-2-deoxycytidine (CdR), a potent DNAMeTase inhibitor.
HPLC analysis indicated that HCA2 cells (PD 28) incubated for 5 days (2-3 doublings) with 0.5 uM CdR (see methods) exhibited rapid demethylation, and levels decreased to those observed in senescent cells (figure 1A).

**DNAMeTase inhibition induces growth arrest in HCA2 cells.** We analyzed the phenotype of the CdR treated cells by measuring tritiated thymidine incorporation and cell number in the culture. Cell counts at different times during and after the CdR treatment indicated that the demethylation was accompanied by cessation on culture growth (figure 2A). Quantitation of labeled nuclei in HCA2 cells, incubated for 48 hours in the presence of tritiated thymidine at the end of the treatment, confirmed this observation and suggested that the cells withdraw from the cell cycle (figure 2B). The cell cycle profile of the treated cells by flow cytometry demonstrated that the CdR treated cells were arrested both in G1 and G2, resembling normal fibroblasts in late senescence (table 1).

The loss of proliferative capacity induced by the DNAMeTase inhibitor was irreversible, as the cells did not re-enter the cell cycle for at least up to 3 weeks after removal of the drug. Treatment of normal young cells made quiescent by serum starvation with the cytosine analogue did not affect the ability of the cells to respond to serum stimulation (data not shown). This demonstrates that incorporation of CdR into DNA is necessary for DNAMeTase inhibition and induction of growth arrest.

To determine the specificity of the response, we exposed the cells to DNAMeTase antisense oligonucleotides (28). We electroporated HCA2 cells with 100 nM antisense oligonucleotides every other day for 5 days. Four days after the final electroporation we plated them at a density of 20 cells per 60-mm dish. This treatment resulted in a decrease in the colony size distribution, a measure of the growth ability that was not observed in cells treated with an unrelated
phosphorothioate oligonucleotide (figure 3). This indicates that the growth arrest is not due to pleiotropic effects of the CdR inhibitor but is rather induced by inhibition of DNAMeTase.

**DNAMeTase inhibition-induced growth arrest has many senescent cell-like features.** To further characterize the CdR-induced proliferative arrest we tested some of the known senescence-associated changes in the arrested cells. CdR treated cells acquired a senescent-like morphology, increased size and expressed the senescence-associated β-galactosidase activity (figure 4A). Northern analysis of the expression of type I collagenase, a gene known to be overexpressed in senescence, revealed that collagenase expression was induced in HCA2 cells after CdR treatment (figure 4B).

The hallmark of cellular senescence is the inability of senescent cells to be stimulated to synthesize DNA by the addition of mitogens or serum. The only agent that overcomes this terminal arrest is SV40 T antigen (37). In order to test the ability of CdR-arrested cells to re-enter the cycle upon T antigen introduction, we analyzed tritiated thymidine incorporation of cells in which the T antigen expression plasmid (PSV7) had been introduced. HCA2 senescent (PD 88) and CdR treated cells (9 days post-treatment) were electroporated with the PSV7 plasmid and 24 hours later incubated for 48 hours in the presence of tritiated thymidine. We quantitated the number of cells that expressed T antigen (positive for immunocytochemistry using an anti-T antigen antibody), the number that synthesized DNA (TdT labeled nuclei) and the number that exhibited both features. T antigen expression induced both senescent and CdR-arrested HCA2 cells to synthesize DNA (figure 5). As expected, untransfected cells (not stained by the antibody) remained arrested (figure 5).

**P21 is required for the growth arrest mediated by inhibition of DNAMeTase.** We further characterized the arrested phenotype by analyzing the pattern of expression of cell cycle proteins.
Western analysis of CdR-treated HCA2 cells showed no changes in p53, p33, p27 or p16 immunoreactivity (figure 6A). However, there was a 5-6 fold increase in p21 protein levels following CdR treatment (figure 6A). Northern analysis indicated that the increase in p21 protein was accompanied by an elevation of p21 mRNA levels (figure 6C). The increase in p21 mRNA was evident after 1 population doubling in the presence of the drug (fig 6C, day 2). p21 protein was elevated after 2 PD and continued to increase until the end of the treatment (figure 6B).

In order to identify the molecules involved in the cell cycle arrest caused by inhibition of DNAMeTase, we treated a series of cell lines bearing mutations in known cell cycle regulators. These included cells lacking a functional p53 (T98G, Susm1, EJ, SaOS-2, 041), Rb (Hela, Susm-1, SaOS-2, J82,) and p16 (T98G, 041, HT1080, MCF7, HCT116). 0.5 µM CdR treatment for 2-3 PD in culture induced cell cycle arrest in all the cell lines analyzed independent of the mutation, indicating that these proteins are not required for the CdR-induced senescent-like state (data not shown). Nevertheless, a subset of these cell lines that includes CMV, EJ, O41 and HCT116 cells, exhibited no p21 induction after the treatment. These cell lines have been reported to have the p16 promoter hypermethylated and therefore expression of the gene is repressed. As expected, they displayed a very strong re-expression of p16 upon treatment with CdR (figure 6A).

We next examined the significance of the p21 gene in the arrest induced by inhibition of DNAMeTase. For this purpose, we used the parental normal human diploid lung fibroblast cell line LF1, along with H07.2-1 cells, in which the p21 gene has been deleted by double homologous recombination. CdR treatment induced LF1 to enter a senescent-like state, with kinetics similar to that observed for HCA2 (normal fibroblasts derived from neonatal foreskin). These cells also showed an early induction of p21 mRNA followed by an increase in p21 protein levels (data not shown). In contrast, there was no effect of the CdR treatment on the cell cycle of the H07.2-1 cells, measured by 3H-TdR incorporation (figure 7) and cell number (data not shown). To rule out the
possibility of differential incorporation of the drug into the DNA, or altered DNAMeTase response in the p21−/− cells, we measured genomic DNA methylation levels in the CdR treated cells. HPLC analysis indicated that inhibition of DNAMeTase by CdR treatment decreased global methylation levels in H07.2-1 cells from 2.46 ± 0.42 % to 1.8 ± 0.4 % (figure 8). Taken together, these data suggest that that the DNAMeTase inhibition-induced arrest is mediated by p21 and is not dependent on p53, Rb or p16.

Interestingly, the 5mC content of the H07.2-1 cells was lower than that of senescent cells (figure 8). These cells had bypassed senescence and were in an extended life span period (23). The decrease in methylation levels relative to the parental cells and to levels below that of senescent fibroblasts suggest that the subcultivation related demethylation persists in cells that have circumvented senescence.

Expression of antisense p21 mRNA eliminates DNAMeTase inhibition-mediated growth arrest. The causal association between p21 and the DNAMeTase inhibition-induced arrest was further corroborated in HCA2 cells. We determined the effect of antisense p21 mRNA expression on the ability of DNAMeTase inhibition to halt proliferation in HCA2 cells. Expression of antisense p21 mRNA decreased p21 protein to undetectable levels (figure 9A) and has been shown to abolish p21-mediated growth arrest in cotransfection assays (38). Cotransfection of CMV-AS-p21, expressing antisense p21 mRNA, +1 to +165, with CMV-EGFP into HCA2 cells was followed by addition of CdR or control medium 3 hours after plasmid delivery. CdR treatment was continued for 7 days. Expectedly, cells receiving control DNA (empty vector) exhibited a marked decrease in DNA synthesis, assayed as thymidine incorporation. 96% of CdR treated cells failed to incorporate tritiated thymidine (data not shown), regardless of GFP expression. In contrast, the expression of antisense p21 mRNA abolished CdR induced growth arrest. 85% of GFP expressing cells (presumably expressing antisense p21 mRNA) exhibited thymidine incorporation
(figure 9B). GFP positive cells that are still responsive to CdR treatment (15%), may represent a subpopulation of cells transfected only with the GFP construct, or that express low levels of antisense p21 mRNA. On the other hand, only 3% of cells negative for GFP expression synthesized DNA (figure 9B). These data support the conclusion that DNAMeTase inhibition induces growth arrest through the p21 protein.

DISCUSSION

The results presented here indicate that inhibition of DNAMeTase in normal diploid human fibroblasts, as well as in a variety of human immortal cell lines, results in an irreversible arrest in cell proliferation. The arrest occurred when either CdR or DNAMeTase antisense oligonucleotides were used, indicating that the inhibition of replication is not a consequence of the pleiotropic effects of CdR. The arrested phenotype exhibited many characteristics of a senescent like state, including cell morphology, DNA content, expression of the SA-β–galactosidase activity, induction of type I collagenase and p21 expression. In addition, the DNA synthesis inhibition could be overcome by expression of the SV40 T antigen, a hallmark of senescent cells. The data indicate that DNAMeTase inhibition causes growth arrest in normal human fibroblasts by upregulation of the cell cycle inhibitor p21. p21 induction appears to be essential for the growth arrest to occur, as p21 minus cells and cells expressing antisense p21 mRNA are refractory to DNAMeTase inhibition. The lack of induction of SA β-gal activity and type I collagenase expression in p21−/− CdR-treated cells suggests that these are downstream effects of p21 induction. Alternatively, they could be manifestations of the arrested phenotype. We favor the latter possibility as cells arrested by serum starvation express high levels of p21 but do not exhibit any of these markers. A similar absolute requirement of p21 for growth arrest has been shown in response to other stimuli, including histone deacetylase inhibition, γ-irradiation and BRCA1 overexpression (39, 40, 41). Conversely, p21 is not essential in the growth arrest induced by serum starvation (our data and 39).
Levels of the tumor suppressor p53 were not modified by DNAMeTase inhibition and p21 upregulation and cell cycle arrest after DNAMeTase inhibition were also observed in cell lines lacking a functional p53. This suggests that p21 upregulation in this case is most likely to be independent of p53. This further supports the idea that p21 is upregulated upon DNAMeTase inhibition by a p53 independent mechanism. Recently, it has been reported that inhibition of DNAMeTase by DNAMeTase antagonists or antisense oligonucleotides results in p21 induction with a concomitant inhibition of DNA replication (28, 29). Our results suggest that transcriptional activation accounts for p21 induction after DNAMeTase inhibition as p21 mRNA levels are rapidly increased after treatment. The demonstration that DNAMeTase has a transcriptional repression domain and associates with histone deacetylase activity and the co-repressor DMAP1 suggests that p21 transcriptional activation resulting from DNAMeTase inhibition involves derepression of the p21 promoter (30, 32). In addition, p21 expression is induced by HDAC inhibitors through a process involving histone hyperacetylation (42). Thus, the molecular mechanism for DNAMeTase repression of p21 could involve recruitment of histone deacetylase activity to the p21 promoter.

The observed proliferation inhibition was also independent of Rb and p16 status, as cell lines deficient in these proteins exhibited cell cycle withdrawal and p21 induction after DNAMeTase inhibition. This suggests that DNAMeTase repression of p21 is not mediated by interaction with the repressive DNAMeTase-Rb-E2F1 complex (31).

In spite of their similar phenotype after CdR treatment, cell lines that had p16 inactivated by methylation showed a different pattern of immunoreactivity of cell cycle regulatory proteins in response to DNAMeTase inhibition from cell lines in which p16 was not methylated. Western analysis indicated that cells reported to have the p16 promoter methylated (CMV, EJ, O41, HCT116) and therefore repressed, produced high levels of the protein after CdR treatment, suggesting promoter demethylation. The level of p16 immunoreactivity present in these cells after
demethylation was much higher than that observed in other cell lines, normal senescent cells or p21<sup>−/−</sup> cells that had overcome senescence and thus had high p16 levels. This observation suggests that methylation of the p16 promoter introduces a modification of chromatin structure that not only limits accessibility of transcriptional activators, but also prevents basal repressors from acting on the p16 gene. The relief of these constraints by inactivation of DNAMeTase allows the transcriptional machinery to gain preferential access to the promoter region, leading to deregulated gene expression. High expression of p16 per se could induce the observed growth arrest in this subset of immortal cell lines with p16 promoter hypermethylated.

The incorporation of the CdR base analogue into the genomic DNA traps DNAMeTase covalently, resulting in inhibition of its activity. Therefore, the observed arrest could be attributed to an interference of this adduct in the DNA replication process. However, this possibility seems to be ruled out by the T antigen induced DNA synthesis data and the observation that cells lacking the p21 protein (p21<sup>−/−</sup> or antisense p21 expressing cells) can proliferate normally in the presence of the drug.

Several lines of evidence now indicate that normal cells have a mechanism that permits the counting of cell divisions. Alteration of such a mechanism is important and necessary for immortalization. The data presented here suggest that DNAMeTase activity and/or DNA methylation levels could be the signals that trigger the senescence response to replicative aging in human fibroblasts. As is the case with global DNA methylation levels, DNAMeTase activity was shown to decrease in an age dependent manner (21). This observation supports the idea that this protein is involved in the division counting mechanism. However, it remains to be determined whether the DNAMeTase participation in the determination of cellular life span is mediated by its methyltransferase activity and/or its capacity for methylation-independent transcriptional regulation.
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Table 1. The decline in growth rate was coupled to an increase in the proportion of cells in the G2 phase of the cell cycle. FACS analysis (propidium iodide cytofluorometry), followed by computer determination of the percentage of cells in the different phases of the cell cycle was carried out on control cells at 25 and 80 PD and on CdR (0.5 µM) treated cells at 25 PD.
FIGURE LEGENDS

**Figure 1.** Global DNA methylation levels and DNAMeTase activity decrease as cells age in culture and in 5-aza-2-deoxycytidine (CdR) treated cells. A. Genomic methylation levels were measured in HCA2 cells at the indicated PD by reverse phase HPLC at a pH of 4.5. The percentage of methylated cytosine was determined after enzymatic hydrolysis. In the case of CdR treated cells (PD28 CdR, solid bar) 0.5 µM CdR was added to the culture medium every day for 5 days and the cells harvested 48 hr after the last CdR addition. B. DNAMeTase activity was measured at the indicated PD using an hemimethylated substrate as described in materials and methods. The percentage of dividing cells, analyzed by ³H thymidine incorporation (48 hr) is indicated inside bars.

**Figure 2.** DNAMeTase inhibition induces cell cycle arrest. A. The effect of 0.5 µM CdR treatment on the growth of young human fibroblasts. HCA2 cells (PD 25) were seeded at a density of 10⁴ cells/cm² and split at the same density every 3 days. An aliquot of cells was counted at each split to compute the PD achieved. B. Cells were cultured for 24 hr in the presence of tritiated thymidine ([³H] TdR) 3 days after the last addition of CdR. The percentage of labeled nuclei before (light bars) and after (solid bars) CdR treatment was scored microscopically. Control cultures had been treated the same way as CdR cultures, but no drug was added to the medium.

**Figure 3.** Treatment of normal fibroblasts with DNAMeTase antisense oligonucleotide induces cell cycle arrest. HCA2 cells were electroporated with MG88, a DNAMeTase antisense oligonucleotide (solid bars) or with an unrelated oligonucleotide (light bars) and split at a density of 20 cells/60 mm dish. Two weeks later the colonies were fixed and stained with crystal violet and the number of cells per individual colony was determined by bright field microscopy.
**Figure 4.** CdR treatment induces a senescent like morphology and expression of senescent associated proteins. A. Photomicrographs of HCA2 control and 0.5 µM CdR treated cells stained for β-galactosidase activity at pH 6 (35). B. HCA2 cells were seeded at standard density (10^4/cm^2) and 24 hr later treated with 0.5 µM CdR for 7 days. Steady state mRNA levels of collagenase were examined by northern blot analysis. Probing the filter for β-actin was used to determine equal loading.

**Figure 5.** The block in DNA synthesis induced by DNAMeTase inhibition can be reversed by SV40 large T antigen. CdR treated (PD 25) and senescent HCA2 cells were electroporated with pSV7 and maintained for 24 hr in medium containing [3H] thymidine. Cells were fixed and processed for the simultaneous detection of T antigen immunoreactivity and thymidine incorporation. Light bars: percentage of labeled nuclei in cells not expressing T antigen. Solid bars: percentage of labeled nuclei in cells expressing T antigen.

**Figure 6.** CdR treatment induces changes in p21 mRNA and protein levels. A. Protein extracts (20 µg) obtained from control (lanes 1 and 3) or treated with 0.5 µM CdR (lanes 2 and 4) HCA2 cells (PD 27) (lanes 1 and 2) and O41(lanes 3 and 4) cell were subjected to western analysis for the immunodetection of p53, p33, P21, p16 and actin as loading control. B. Cells were harvested at indicated times (days) after the first addition of 0.5 µM CdR and p21 immunoreactivity was analyzed by western blot. C. Total RNA from cells treated for the indicated time (1-5 days after the first addition of the drug) with 0.5 µM CdR was subjected to northern blot analysis for the determination of p21 mRNA levels.
**Figure 7.** DNAMeTase inhibition-induced cell cycle arrest is dependent on the presence of p21. HO7.2-1 (p21<sup>−/−</sup>) cells were split at a density of 10<sup>4</sup> cells/cm<sup>2</sup> 24 hr before the addition of 0.5 µM CdR. Media were changed every day for media containing fresh CdR for 14 days. [³H] thymidine incorporation was measured for 24 hr at the end of the treatment. Light bars indicate the percentage of labeled nuclei before the addition of CdR. Solid bars indicate the percentage of labeled nuclei observed at the end of CdR treatment. Control cultures had been treated the same way as CdR cultures, but no drug was added to the medium.

**Figure 8.** CdR treatment induces demethylation in p21<sup>−/−</sup> cells. Genomic DNA from young and senescent p21<sup>+/+</sup> (LF1, light bars) and p21<sup>−/−</sup> (HO7.2-1, solid bars) cells control (control) and CdR treated (CdR) cells was subjected to HPLC analysis for the determination of DNA methylation levels.

**Figure 9.** p21 protein is responsible for CdR inhibition of DNA synthesis. A. Expression of antisense p21 mRNA decreases p21 protein levels. Increasing amounts (0.0 to 1.5 µg, as indicated) of the CMV-AS-p21 construct (expressing antisense p21 mRNA) was cotransfected with 0.25 µg CMV-p21 (expressing p21) into HCA2 cells and 48 hr later cells were harvested and subjected to western analysis for the immunodetection of p21 and actin. B. Expression of antisense p21 mRNA blocks CdR-induced DNA synthesis inhibition. HCA2 cells (PD 19) were cotransfected with CMV-EGFP (0.7 µg) and CMV-AS-p21 (1.5 µg) plasmids. Transfection medium was replaced 3 hr after plasmid delivery with medium containing 0.5 µM CdR. Media were changed every day for media containing fresh CdR for 7 days. [³H] thymidine incorporation was measured for 24 hr at the end of the treatment. Photomicrographs of two representative fields under UV light (left panel, taken before autoradiographic development), phase contrast and bright field superimposed (middle
panel) and bright field (right panel). Black arrows in middle panel indicate untransfected cells (GFP negative) arrested by the CdR treatment, black arrowheads denote transfected cells (GFP positive) with labeled nuclei and white arrowheads indicate transfected cells (GFP positive) that are nevertheless arrested.
Figure 1
Figure 2
Figure 3
Figure 4

A

Control

CdR (0.5 μM)

B

Collagenase

Actin

CdR

CdR
Figure 5
Figure 7
Figure 8
Figure 9
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DNA methyltransferase inhibition in normal human fibroblasts induces A p21 dependent-cell cycle withdrawal
Juan I. Young and James R. Smith

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