MOLECULAR AND CELLULAR BIOLOGY, Dec. 1992, p. 5536–5540
0270-7306/92/125536-05$02.00/0
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Vol. 12, No. 12

A Mammalian Cell Line Deficient in Activity of the DNA Repair Enzyme 5-Hydroxymethyluracil-DNA Glycosylase Is Resistant to the Toxic Effects of the Thymidine Analog 5-Hydroxymethyl-2′-Deoxyuridine

ROBERT J. BOORSTEIN,* LAI-NO CHIU, AND GEORGE W. TEEBOR

Department of Pathology and the Rita and Stanley Kaplan Cancer Center, New York University Medical Center, 550 First Avenue, New York, New York 10016

Received 31 July 1992/Returned for modification 30 August 1992/Accepted 14 September 1992

We isolated a mutant mammalian cell line lacking activity for the DNA repair enzyme 5-hydroxymethyl-uracil-DNA glycosylase (HmUra-DNA glycosylase). The mutant was isolated through its resistance to the thymidine analog 5-hydroxymethyl-2′-deoxyuridine (HmdUrd). The mutant incorporates HmdUrd into DNA to the same extent as the parent line but, lacking the repair enzyme, does not remove it. The phenotype of the mutant demonstrates that the toxicity of HmdUrd does not result from substitution of thymine in DNA by HmUra but rather from the removal via base excision of large numbers of HmUra residues in DNA. This finding elucidates a novel mechanism of toxicity for a xenobiotic nucleoside. Furthermore, the isolation of this line supports our hypothesis that the enzymatic reparability of HmUra derives not from its formation opposite adenine via the oxidation of thymine, but rather from its formation opposite guanine as a product of the oxidation and subsequent deamination of 5-methylcytosine.

The pyrimidine base 5-hydroxymethyluracil (HmUra) is a normal constituent of the DNA of several *Bacillus subtilis* bacteriophages and dinoflagellate species, performing the base pairing function normally reserved for thymine (19). However, a repair enzyme, HmUra-DNA glycosylase, is present in most higher organisms which removes this modified base from DNA (2, 3, 6, 9, 10, 15). Analogously, uracil replaces thymine in the DNA of other *B. subtilis* phages (27) but is removed from DNA via the action of uracil-DNA glycosylase, which is present in an even wider spectrum of eukaryotic and prokaryotic organisms (12).

Uracil is formed in cellular DNA in one of two ways: (i) through the incorporation of dUTP in lieu of TTP, producing uracil opposite adenine, or (ii) via the deamination of cytosine, resulting in uracil opposite guanine. The latter mechanism accounts for the reparability of uracil, since a uracil opposite a guanine would be mutagenic if not repaired (12).

HmUra may also be formed in DNA by two analogous pathways. The first is via oxidative attack on the methyl group of thymine resulting in formation of an HmUra opposite adenine (11, 28). The second is via deamination of 5-hydroxymethylcytosine, which could be formed in DNA as a consequence of oxidative attack on the methyl group of 5-methylcytosine (1, 14). This would result in an HmUra opposite guanine. Since HmUra pairs as thymine and/or uracil (23), its location opposite guanine would similarly be mutagenic if not repaired. We have previously proposed that HmUra-DNA glycosylase has evolved to repair HmUra formed by the latter mechanism (2, 5, 10).

However, the administration of the nucleoside 5-hydroxymethyl-2′-deoxyuridine (HmdUrd) to cells grown in culture and to laboratory animals results in significant toxicity (18, 20, 25, 32). Since HmdUrd is incorporated into DNA as a thymidine analog, its cytotoxicity suggested that the formation of HmUra opposite adenine is deleterious to the cell. We have administered HmdUrd to mammalian cells growing in culture and confirmed that toxicity occurred but only when the level of substitution of thymidine by HmUra exceeded 0.1% (4). This finding led us to hypothesize that the toxicity of HmdUrd did not result from substitution of thymine with HmUra but rather from the repair of large numbers of HmUra residues in cellular DNA (4).

If this hypothesis is correct, then cells unable to remove HmUra from DNA via base excision repair should be resistant to the toxic effects of HmdUrd. To test this hypothesis, V79 Chinese hamster cells which are normally HmdUrd sensitive and express HmUra-DNA glycosylase activity were mutagenized, and colonies resistant to HmdUrd were isolated, expanded, and analyzed to determine whether they expressed HmUra-DNA glycosylase activity.

MATERIALS AND METHODS

Materials. [^{3}H]thymidine was purchased from Dupont, NEN Research Products, while [^{3}H]HmdUrd was synthesized (23) and purified according to standards previously described (4). Other chemicals were purchased from Sigma, unless otherwise stated.

Cell culture. Stocks of V79 cells and cell strains derived from V79 cells were maintained as described previously (4) in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (DME).

Development and isolation of HmdUrd-resistant Chinese hamster cells. HmdUrd-resistant Chinese hamster cell mutants were obtained from V79 cells by a modification of the Gene-Tox protocol (7). A total of 2×10^7 cells per experiment were treated with 10 μM *N*′-methyl-*N*-nitro-*N*-nitrosoguanidine to produce 60 to 90% toxicity. To permit expression and fixation of mutations, cells were grown for 7 days after removal of the mutagen, during which time they were subcultured twice. The cells were then plated in selective medium (10 μM HmdUrd, 2×10^6 cells per 100-mm plate, 10

* Corresponding author.

plates per experiment). Approximately 60 HmdUrd-resistant colonies were isolated and subcultured under selective conditions. Colonies that grew well were further expanded under selective conditions. The goal was to obtain a mutant cell line that was resistant to HmdUrd while retaining the growth characteristics of the parent V79 cells. From a series of three experiments (6×10^7 cells), one colony, designated V79*mut*1, with the phenotype described below was isolated. Two additional colonies met the criteria of good growth in selective medium. However, these colonies failed an initial screen since they did not incorporate normal amounts of HmdUrd. Such colonies were presumed to have no abnormalities in HmUra-DNA glycosylase activity and were not characterized further.

Assay of cell growth of V79 and V79*mut*1 cells in the presence of HmdUrd. V79 cells or V79*mut*1 cells were plated in duplicate at a concentration of 5×10^4 cells per 35-mm dish in complete DME. After 24 h (time = 0), 0 to 10 μM HmdUrd was added. After a second 24-h period, cells were washed and placed in fresh medium. After a third 24-h period, the cells were counted as described previously (4). During the entire experiment, the cells were subconfluent and proliferating.

Measurements of colony-forming ability. V79 and V79*mut*1 cells were plated in duplicate in complete medium at a density of 5×10^4 cells per 35-mm dish. After 24 h, HmdUrd was added, and the cells were incubated for an additional 24 h. The cells were then replated in complete medium at low density (100 cells per 60-mm dish) in triplicate, and colony-forming ability as a percentage of untreated controls was determined after 10 days of further growth as previously described (4). Control plating efficiency was 82% for V79 cells and 66% for V79*mut*1 cells.

Measurement of [^{3}H]thymidine and [^{3}H]HmdUrd incorporation in V79 and V79*mut*1 cells. V79 and V79*mut*1 cells were plated in complete medium in duplicate at densities of 5×10^4 cells per 35-mm dish. After 24 h, 0.1 or 1 μM thymidine or HmdUrd was added to the medium along with 0.1 μCi of [^{3}H]thymidine or 1 μCi of [^{3}H]HmdUrd. After 24 h of growth in this medium, the amount of radioactivity incorporated into trichloroacetic acid (TCA)-precipitable material was determined. For each condition, the net incorporation of nucleoside in picomoles was determined from the calculated specific activities of the radioactive nucleosides in the medium. We have previously demonstrated that [^{3}H]HmdUrd is incorporated into the DNA of mammalian cells without modification (4).

Measurement of specific repair of [^{3}H]HmdUrd incorporated into DNA. The incorporation of [^{3}H]HmdUrd into DNA and the loss of [^{3}H]HmdUrd-derived radioactivity was determined as described previously (4). V79 and V79*mut*1 cells were plated (2×10^4 per 35-mm dish). After 24 h, 1 μM thymidine containing 0.005 μCi of [^{14}C]thymidine was added to the medium to uniformly prelabel cellular DNA. After 24 h of growth in the presence of [^{14}C]thymidine, the cells were washed with complete DME, and 0.1 μM HmdUrd containing 0.5 μCi [^{3}H]HmdUrd was added in fresh medium. After a third 24-h period, the medium containing [^{3}H]HmdUrd was removed. The cells were then washed twice with complete DME, incubated for 30 minutes, and washed an additional time with complete DME. The cells were then reincubated in complete medium. After a fourth 24-h period, the medium was removed, TCA-precipitable ^{3}H and ^{14}C radioactivity was determined, and the loss of ^{3}H radioactivity (HmUra) relative to ^{14}C radioactivity (thymine) was calculated. The loss of ^{14}C radioactivity with time was considered to result from cell death, while the loss of ^{3}H radioactivity relative to ^{14}C radioactivity was considered to result from repair.

In these experiments, the amount of incorporated ^{14}C declined less than 10% during the experiment. Control cells pretreated with [^{14}C]thymidine but not treated with [^{3}H]HmdUrd lost a similar amount of TCA-precipitable ^{14}C radioactivity during the same period. In three experiments done in duplicate, the final amount of TCA-precipitable ^{14}C radioactivity was 1,570 dpm in V79 cells and 1,730 dpm in the V79*mut*1 cells. Therefore, we concluded, as we had previously (4, 6), that the loss of ^{3}H from these cells reflected the specific removal of [^{3}H]HmUra from DNA containing [^{3}H]HmdUrd.

Measurement of DNA glycosylase activities. Cellular extracts of 5×10^6 to 10×10^6 V79 cells and V79*mut*1 cells were prepared and assayed for the presence of HmUra-DNA glycosylase and uracil-DNA glycosylase using techniques described previously (3). Briefly, cells were washed and sonicated, extracts were prepared, and protein concentrations were determined (3). Extracts were then incubated with either [^{3}H]HmUra-DNA from SPO1 phage (50,000 to 100,000 dpm/μg) or [^{3}H]uracil-DNA from PBS2 phage (100,000 to 200,000 dpm/μg). The amount of [^{3}H]HmUra or [^{3}H]uracil released was then determined by high-pressure liquid chromatography (HPLC) as described previously (2, 3, 10).

TABLE 1. Effect of HmdUrd on growth of V79 and V79*mut*1 cells[a]

HmdUrd added (μM)	V79		V79*mut*1	
	Growth (%)	Doublings	Growth (%)	Doublings
0	296	1.99	388	2.29
0.1	356	2.19	374	2.24
1	135	1.23	474	2.52
10	−9	−0.14	383	2.27

[a] V79 cells or V79*mut*1 cells were treated with 0, 0.1, 1, or 10 μM HmdUrd for 24 h as described in Materials and Methods. Twenty-four hours after the HmdUrd was removed, the cell number per dish was determined. The percent increase in cell number and the calculated number of cell doublings in the 48-h interval from the time of drug addition until cell number determination is indicated. Each point represents the average cell number of two identically treated dishes.

RESULTS

Growth characteristics of V79 and V79*mut*1 cells. To characterize the HmdUrd resistance of the mutant strain, we compared V79 and V79*mut*1 cells for their ability to grow in the presence of increasing concentrations of HmdUrd (Table 1). The V79 cells did not grow at 10 μM HmdUrd and were inhibited in their growth at 1 μM HmdUrd. The growth rate of V79*mut*1 cells, in contrast, was not affected by 10 μM HmdUrd. In the absence of HmdUrd, both cell lines grew at similar rates.

Colony-forming ability of V79 and V79*mut*1 cells. To assess the effect of HmdUrd on colony-forming ability, we exposed V79 and V79*mut*1 cells to increasing concentrations of HmdUrd and replated them at low density (Fig. 1). The V79 cells showed markedly reduced colony-forming ability at concentrations of 1 to 10 μM HmdUrd, while the V79*mut*1 cells were resistant to the toxic effects of HmdUrd.

Incorporation of nucleosides by V79 and V79*mut*1 cells. The ability of cells to incorporate exogenously added nucleosides was determined to demonstrate that the mutant phenotype

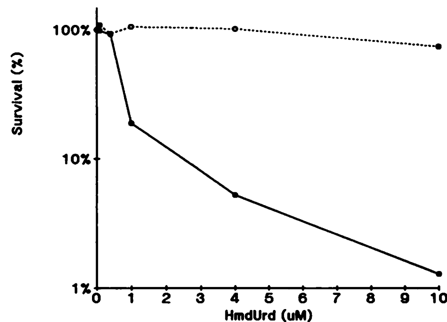

FIG. 1. Measurements of colony-forming ability. V79 (●) and V79*mut*1 (○) cells were grown in the presence of HmdURd, and colony-forming ability as a percentage of untreated controls was determined as previously described (19). Each point represents the average plating efficiency of three dishes from a representative experiment.

TABLE 2. Incorporation of HmdUrd and thymidine into V79 and V79*mut*1 cells[a]

Nucleoside added (μM)	Nucleoside incorporated (pmol ± SD)		Ratio of incorporation (HmdUrd/thymidine)
	HmdUrd	Thymidine	
V79			
0.1	0.88 ± 0.04	27.9 ± 0.35	0.031 ± 0.001
1	10.5 ± 0.97	308 ± 9.32	0.034 ± 0.001
V79*mut*1			
0.1	0.71 ± 0.07	25.6 ± 0.17	0.028 ± 0.002
1	8.56 ± 0.14	282 ± 28.0	0.030 ± 0.002

[a] V79 and V79*mut*1 cells were plated in complete medium in duplicate. After 24 h, 0.1 or 1 μM thymidine or HmdUrd was added to the medium along with 0.1 μCi of [^{3}H]thymidine or 1 μCi of [^{3}H]HmdUrd. After 24 h, the amount of radioactivity incorporated into TCA-precipitable material was determined. Each point represents the average of two determinations.

did not result from failure to incorporate either 2′-deoxynucleosides in general or HmdUrd in particular. Cells were exposed to either [^{3}H]HmdUrd or [^{3}H]thymidine for 24 h, and net incorporation was measured. The V79 and V79*mut*1 cells showed similar net incorporation of both nucleosides (Table 2).

Repair of HmUra by V79 and V79*mut*1 cells. To determine whether the mutant cells were deficient in their ability to remove HmUra from their DNA, cells were prelabelled with [^{14}C]thymidine and then grown in [^{3}H]HmdUrd. The loss of ^{3}H-containing material from cellular DNA relative to ^{14}C-containing material was measured (4). Three determinations done in duplicate showed the fractional loss of ^{3}H-containing material in 24 h to be 0.35 ± 0.01 in the V79 cells and 0.06 ± 0.07 in the mutant cells. Thus, repair of HmUra in vivo is markedly diminished and probably completely absent in the mutant cells.

One might ask, if the repair of HmUra is deficient in the mutant cells, why was there no significant difference in net incorporation of HmdUrd between V79 and V79*mut*1 cells as shown in Table 2? Net incorporation equals the amount of HmdUrd incorporation minus that removed via repair during the 24-h period. Incorporation of HmdUrd took place throughout the entire period during which the cells were growing in an exponential fashion. Assuming uniform incorporation into DNA, at the end of 24 h, the average HmdUrd molecule would have been in DNA for about 8 h. We have shown that, at these levels of substitution, about 30% of the HmUra residues are removed from DNA in 24 h (4). Thus, during 8 h, only 10% of the HmUra would be removed. This 10% difference between the parent strain and the mutant cell line is too small to be detected reliably by measuring net incorporation. For this reason, we used the more specific ^{14}C prelabelling technique for measuring repair.

Measurement of DNA glycosylase activities in V79 and V79*mut*1 cells. Extracts of V79 and V79*mut*1 cells were assayed for HmUra-DNA glycosylase activity (3, 9, 10) to determine whether the failure on the part of the mutant cell line to repair HmUra resulted from loss of HmUra-DNA glycosylase activity. Uracil-DNA glycosylase activity was assayed in parallel as a control for the adequacy of the preparation of cellular extracts. The specific activity of uracil-DNA glycosylase was the same in extracts of V79 and V79*mut*1. In sharp contrast, HmUra-DNA glycosylase activity could not be detected in extracts from mutant cells (Fig. 2).

Stability of the V79*mut*1 phenotype. The V79*mut*1 cells were maintained in continuous culture for 9 months in both the presence and the absence of selection with 10 μM HmdUrd. After this interval, the mutant cells grown under

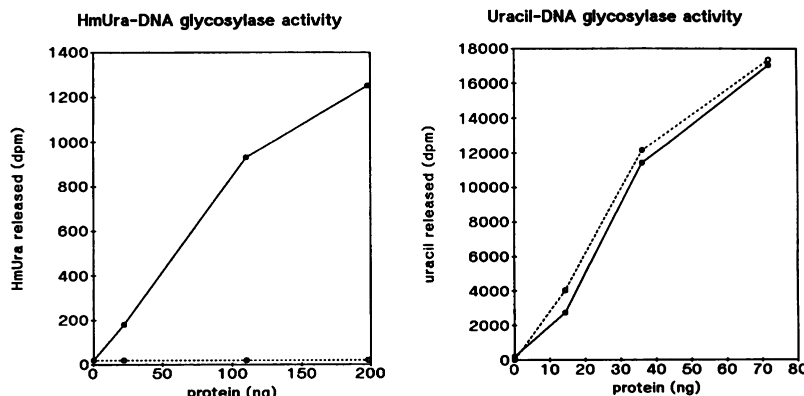

FIG. 2. Measurement of DNA glycosylase activity. Cellular extracts of 5 × 10^6 to 10 × 10^6 V79 cells (●) and V79*mut*1 cells (○) were prepared and assayed for the presence of HmUra-DNA glycosylase and uracil-DNA glycosylase by techniques described previously (3). The amount of [^{3}H]HmUra or [^{3}H]uracil released was then determined by HPLC. Data are from a representative experiment.

both selective and nonselective conditions remained resistant to HmdUrd and did not express detectable levels of HmUra-DNA glycosylase activity.

DISCUSSION

The phenotype of the V79*mut*1 cells that we isolated is HmdUrd resistance, HmdUrd incorporation positive, and HmUra-DNA glycosylase deficiency. The phenotype proved stable, remaining unchanged after 9 months of continuous culture in the presence or absence of continued selection. This V79*mut*1 cell strain is phenotypically distinct from other HmdUrd-resistant cell lines, all of which are characterized by a deficiency in HmdUrd incorporation without an alteration in HmUra-DNA glycosylase activity (3, 20, 21, 32).

These experiments provide evidence that the toxicity of HmdUrd does not result from any intrinsic toxic property of HmUra as a substitute for thymine in DNA (25, 32). Previously we showed that V79 cells could tolerate levels of HmdUrd substitution as high as 1 HmUra per 1,000 thymines; however, at higher levels of substitution, toxicity was seen (4). Here we demonstrate, unequivocally, that while HmdUrd must be incorporated into DNA for HmdUrd to be toxic, incorporation without repair is not a sufficient condition for toxicity.

The hypothesis that the toxicity of HmUra in the DNA of mammalian cells results from its repair (4) may, at first glance, seem counterintuitive, since it is generally assumed that the repair of a modified base in DNA prevents that lesion from exerting harmful effects. Nevertheless, there is a precedent for suggesting that excess repair may lead to toxicity. Goulian et al. (13) have suggested that the inhibition of TMP synthesis caused indirectly by methotrexate results in incorporation of relatively large amounts of deoxyuridine into DNA. While the replacement of thymine with uracil is not intrinsically toxic (31), the repair of large amounts of uracil residues via the action of uracil-DNA glycosylase might cause toxicity. Although this mechanism is probably not a major cause of the toxicity of methotrexate, the model is analogous to the one we have suggested here to explain the toxicity of HmdUrd.

The phenotypes displayed by V79 and V79*mut*1 cells when grown in HmdUrd are analogous to the phenotype of *Escherichia coli dut* and *E. coli dut ung* mutants, respectively. *E. coli dut* mutants, defective in dUTPase, incorporate large amounts of uracil into their DNA (30). The uracil is then removed by uracil-DNA glycosylase, and these mutants show a higher than normal amount of recombination (Hyper rec) (30). V79 cells grown in HmdUrd incorporate large amounts of HmUra into their DNA which is repaired by HmUra-DNA glycosylase, resulting in increased numbers of sister chromatid exchanges (22) and reduced viability (4, 6). *E. coli dut ung* mutants also incorporate large amounts of uracil into their DNA. However, the absence of uracil-DNA glycosylase suppresses the Hyper rec phenotype, and the cells tolerate uracil in their DNA without adverse effects (29). V79*mut*1 cells grown in HmdUrd incorporate large amounts of HmUra into their DNA, as do V79 cells. In the absence of HmUra-DNA glycosylase, the HmUra residues persist and the cells grow normally.

We have developed the hypothesis that HmUra-DNA glycosylase evolved to repair HmUra residues that arise opposite guanine. Such residues are products of the deamination of 5-hydroxymethylcytosine (1), which may be formed in DNA as a consequence of oxidative attack on the methyl group of 5-methylcytosine (14). We therefore have proposed that HmUra-DNA glycosylase is one of a group of DNA glycosylases whose function is the maintenance of methylated cytosine residues in DNA (2, 8–10, 17, 33). The biological importance of damage to 5-methylcytosine is underscored by the results of recent studies which indicate that mutagenesis occurs at a disproportionately high rate at cytosines at sites of DNA methylation (16, 26).

Since HmUra pairs as thymine during replication (23), an unrepaired HmUra opposite guanine would result in a G · C-to-A · T transition (1). Thus, the evolution of HmUra-DNA glycosylase may be considered to be analogous to that of uracil-DNA glycosylase. The latter has evolved to prevent G · C-to-A · T transitions resulting from the deamination of cytosine yielding uracil opposite guanine (24). However, uracil-DNA glycosylase also releases uracil from DNA when it is opposite adenine (12), just as HmUra-DNA glycosylase releases HmUra from DNA when it is opposite adenine (3, 10). The failure of these enzymes to distinguish a uracil or HmUra opposite adenine (a match) from one opposite guanine (a mismatch) accounts for the phenotype of the *dut E. coli* mutant and for the phenotype of the parent V79 cells grown in medium containing HmdUrd.

In summary, we isolated a mammalian cell strain resistant to HmdUrd by virtue of loss of HmUra-DNA glycosylase activity. This strain should prove useful in the study of the mechanism by which excessive repair causes toxicity, in elucidating the role of this enzyme in the cellular response to oxidative damage, and in the cloning of the HmUra-DNA glycosylase gene.

ACKNOWLEDGMENTS

This work was supported by an American Cancer Society junior faculty award (R.J.B.) and by NIH grants CA51060, CA49689, CA16669, CA16087, and AM07421.

We are grateful to P. D'Eustachio and L. Loeb for their critical reading of the manuscript and to Archie Cummings and Donna Putnam for technical assistance.

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