Loss of DNA Mismatch Repair in Acquired Resistance to Cisplatin


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ABSTRACT

Selection of cells for resistance to cisplatin, a well-recognized mutagen, could result in mutations in genes involved in DNA mismatch repair and thereby to resistance to DNA-alkylating agents. Parental cells of the human ovarian adenocarcinoma cell line 2008 expressed hMLH1 when analyzed with immunoblot. One subline selected for resistance to cisplatin (2008/A) expressed no hMLH1, whereas another (2008/C13*5.25) expressed parental levels. Microsatellite instability was readily demonstrated in 2008/A cells but not in 2008 and in 2008/C13*5.25 cells. In addition, the 2008/A cells were 2-fold resistant to methyl-nitro-nitrosoguanidine and had a 65-fold elevated mutation rate at the HPRT locus as compared to 2008 cells, both of which are consistent with the loss of DNA mismatch repair in these cells. To determine whether the loss of DNA mismatch repair itself contributes to cisplatin resistance, studies were carried out in isogenic pairs of cell lines proficient or defective in this function. HCT116, a human colon cancer cell line deficient in hMLH1 function, was 2-fold resistant to cisplatin when compared to a subline complemented with chromosome 3 and expressing hMLH1. Similarly, a human endometrial cancer cell line HEC59, which expresses no hMSH2, was 2-fold resistant to cisplatin when compared to a subline complemented with chromosome 2 that expresses hMSH2. Therefore, the selection of cells for resistance to cisplatin can result in the loss of DNA mismatch repair, and loss of DNA mismatch repair in turn contributes to resistance to cisplatin.

INTRODUCTION

DDP\(^1\) forms bifunctional covalent adducts with DNA (1) and is a well-recognized mutagen in mammalian cells (2). It produces mutations at various loci following exposure of cells both in vitro (3) and in vivo (4). Although DDP is a highly effective chemotherapeutic agent for many types of cancer, one concern regarding its use is that its genome destabilizing effect may cause somatic mutations in tumor cells that potentially result in resistance to other drugs or unfavorably alter other characteristics of the malignancy. Loss of DNA mismatch repair also causes destabilization of the genome and is a common finding in a variety of sporadic and inherited human neoplasms (reviewed in Ref. 5), including those for which DDP is routinely used (6–9). It results in high mutation rates, particularly in microsatellite sequences, in both noncoding (10, 11) and coding (12, 13) portions of the genome. In addition, loss of DNA mismatch repair typically results in resistance to the methylating agent MNNG (14), to the antimetabolite 6-thioguanine (15, 16), and possibly to certain types of alkylating agents (17). No information is currently available on the question of whether there is a correlation between the genome-destabilizing effect of DDP and loss of DNA mismatch repair.

In this communication, we demonstrate that treatment of human ovarian carcinoma cells with DDP in vitro at levels of exposure comparable to those attained in patients can induce mutations that functionally alter DNA mismatch repair proteins, and that in turn loss of DNA mismatch repair results in resistance to DDP in two well-described cell lines.

MATERIALS AND METHODS

**Cell Lines.** The human ovarian adenocarcinoma cell line 2008 (18) and the isogenic DDP-resistant sublines 2008/A (19) and 2008/C13*5.25 (20) were maintained in RPMI 1640 supplemented with 2 mM l-glutamine and 10% heat-inactivated fetal bovine serum. 2008/A and 2008/C13*5.25 cells were generated by repeated exposure to escalating concentrations of DDP as described previously (19, 20). The hMLH1-deficient human colorectal adenocarcinoma cell line HCT116 was obtained from the American Type Culture Collection (ATCC CCL 247); sublines complemented with chromosome 3 (HCT116+ch3-6) and chromosome 2 (HCT116+ch2-1) were obtained from Drs. C. R. Boland and M. Koi (21) as were the hMSH2-deficient human endometrial adenocarcinoma cell line HEC59 (22) and a subline complemented with chromosome 2 (HEC59+ch2-4). Both cell lines were maintained in Iscove’s modified Dulbecco’s medium (Irvine Scientific, Irvine, CA) supplemented with 2 mM l-glutamine and 10% fetal bovine serum. The chromosome-complemented lines were maintained in medium supplemented with geneticin (G418; 400 μg/ml for HCT116+ch3-6 and 600 μg/ml for HEC59+ch2-4). The absence and presence of expression of hMLH1 in HCT116 and HCT116+ch3-6 as well as of hMSH2 in HEC59 and HEC59+ch2-4 were verified using the immunoblot analysis (data not shown).

**Materials.** DDP was a gift from Bristol-Myers Squibb Co. (Princeton, NJ). 6-thioguanine, sulforhodamine B, and MNNG were purchased from Sigma Chemical Co. (St. Louis, MO).

**Cytotoxicity Assays.** Cells were seeded into 96-well plates at a density of 5000 (2008) or 6000 (HEC59) cells/well in 100 μl medium. After 24 h, appropriate concentrations of drugs were added in a final volume of 100 μl medium. Control plates were fixed as described below to estimate the cellular protein at time 0 (T0). DDP and 6-thioguanine were diluted directly in medium, whereas the dilutions of MNNG were prepared in DMSO; the volume of DMSO was kept at 0.1% (w/v) at all drug concentrations and in controls. Previous experiments (data not shown) have established that 0.1% DMSO does not affect the viability or growth of these cell lines. After 72 h, growth was stopped by adding 50 μl of 50% (w/v) trichloroacetic acid, and cellular protein was stained with sulfarhodamine B and was measured by spectrophotometry (23). The relative growth rate R was calculated as previously reported (24): If T = T0 + R = (T − T0)(C − T0); if T < T0, R = (T − T0)/T0 with T being the absorbance 72 h after drug treatment, C being the absorbance at 72 h in control untreated wells, and T0 being the absorbance in control wells measured immediately before drug treatment. Each experiment was performed in triplicate. IC50 values were estimated by linear interpolation at r = 0.5.

**HCT116 cells did not yield reproducible results in the sulforhodamine B growth rate assay. Therefore, clonogenic assays were performed by seeding 250 cells of a single-cell suspension into 60-mm plastic dishes. After 24 h, appropriate amounts of DDP were added to the dishes, and the cells were exposed for 1 h. Thereafter, the cells were washed, and new drug-free medium...
was added. Colonies of at least 50 cells were scored visually after 8–10 days. Each experiment was performed in triplicate. IC50 values were estimated using logarithmic interpolation at a relative plating efficiency of 0.5.

**Immunoblot.** Cells were lysed on ice in 150 mM NaCl containing 5 mM EDTA, 1% Triton X-100, 10 mM Tris/HCl (pH 7.4), 0.1 mM phenylmethylsulfonyl fluoride, and 5 mM e-aminocaproic acid. After centrifugation, 100 μg protein were denatured by boiling in an equal volume of 130 mM Tris/HCl (pH 6.8) containing 20% glycerol, 4.6% SDS, and 0.02% bromophenol blue. The proteins were separated using SDS-PAGE on an 8% gel followed by electrophoretic transfer onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). hMLH1 was detected using the monoclonal antibody G168-15 (PharMingen, San Diego, CA), and hMSH2 was detected with the monoclonal antibody AB-1 (Oncogene Science, Manhasset, NY) followed by horseradish peroxidase-conjugated antimouse antibodies (Amersham, Arlington Heights, IL) and generation of chemoluminescence by ECL (Amersham).

**Measurement of the Mutation Rate at the HPRT Locus.** One hundred cells were seeded into ten 35-mm dishes and grown to confluence. The cells were transferred to 150-cm2 flasks and grown to a total cell number of 10 × 10^6. A sample of the cells was used to measure the plating efficiency. The remainder of the cells was split into 10-cm dishes at a density of 5 × 10^5 cells/dish and were exposed to 20 μM 6-thioguanine. The number of resistant colonies was scored visually after 16–20 days. The mutation rates were calculated according to the “method of the mean” of the fluctuation analysis model of Luria and Delbrück (25) and by a modified Galton-Watson analysis model (26). The variance of the mutation rate as measured by fluctuation analysis was estimated according to Kendal and Frost (27).

**Microsatellite Analysis.** Clones of 2008 and 2008/A cell lines were obtained by limiting dilution into 96-well plates. Single cells in a well were allowed to grow and were later transferred into 25-cm2 flasks. DNA was isolated by lysing the cells in 2.6 M NaCl containing 0.133 M EDTA and 0.67% Triton X-100 followed by phenol extraction. For the amplification of microsatellites by PCR, the following pairs of primers were synthesized according to information obtained from the Cooperative Human Linkage Center and the reference sequence (28). EDH17B (5'-GTGACCCACGAAACACAGG-3'), 5'-TTTTATGATGAAAGTATATT-3'). ATA7B (5'-ATGGAAGAGCGTTCTAAAACA-3'), and GATA11B12 (5'-AACAAAAACAAAACAAAACA-3'). Five hundred ng DNA were amplified using 0.2 units Taq polymerase (Boehringer Mannheim) over 40 cycles (94°C, 55°C, and 72°C, 30 s each) in the presence of 2 μCl(3P)-dCTP. The PCR products were separated on a 6% polyacrylamide/8 M urea gel and visualized by using autoradiography.

**RESULTS**

The parental human ovarian cancer cell line 2008 and its DDP-resistant subline 2008/C13*5.25 express hMLH1 and hMSH2 in amounts that are readily detectable with immunoblot (Fig. 1). These cell lines are sensitive to MNNG and to 6-thioguanine. The IC50 for MNNG was 3.3 ± 0.6 μM (SD) for the 2008 cells and 3.5 ± 0.4 μM for the 2008/C13*5.25 cells; for 6-thioguanine, the IC50 values were 6.5 ± 1.13 μM and 3.7 ± 0.3 μM, respectively. These values are similar to those reported for other cell lines (29). Fig. 1 also shows that, in contrast to the 2008/C13*5.25 subline, during the selection for resistance to DDP the 2008/A subline lost the expression of hMLH1 detectable on Western blots. This indicated that a mutation that either blocks the production of message from the hMLH1 gene or results in the synthesis of an unstable truncated protein had taken place, and suggested that these cells should be defective with respect to DNA mismatch repair.

On the basis of previous studies, one would expect the loss of DNA mismatch repair due to disruption of the hMLH1 function to cause resistance to MNNG and 6-thioguanine (29, 30). Fig. 2 shows the growth curves for 2008/A cells exposed to DDP, MNNG, and 6-thioguanine. The 2008/A cells were 60-fold resistant to DDP and proved to be moderately resistant to MNNG as compared to the parental 2008 cells (IC50, 5.0 ± 1.1 versus 3.3 ± 0.6 μM; n = 5, P < 0.05 in a two-sided t test). However, 2008/A cells were unexpectedly 2.6-fold hypersensitive to 6-thioguanine. Thus, the 2008/A cells did not coincide exactly with the anticipated phenotype.

To document further that DNA mismatch repair was defective in the 2008/A cells, 10 single-cell clones were isolated from the 2008/A population and tested for microsatellite instability. Instability was detected at 3 of 8 loci tested (D6S1035, EDH17B, and GGAA2E02) in 2 of 10 clones. Sixteen clones isolated from the parental 2008 cells and 18 clones isolated from the 2008/C13*5.25 population did not show evidence for microsatellite instability when tested with the same panel of eight microsatellite-specific PCR primer pairs. DNA extracted from 8 of the 2008 clones and 5 of the 2008/C13*5.25 clones also failed to demonstrate any microsatellite instability after growth for an additional 5 passages in tissue culture. These results established that the 2008/A cells were in fact defective with respect to DNA mismatch repair function.

Additional confirmation of this defect was sought by determining the mutation rate at the HPRT locus, since loss of DNA mismatch repair has been shown to markedly increase the mutation rate in this gene (12, 31). The mutation rate at the HPRT locus was measured by using fluctuation analysis for the 2008 and the 2008/A cells in parallel. Table 1 shows that the mutation rate was elevated 65-fold in 2008/A cells as compared to the parental 2008 cell line. Thus, on the basis of this parameter also, the 2008/A cells appeared to have a mutator phenotype due to the loss of DNA mismatch repair function. Taken together, these data argue that in the case of the 2008/A subline, selection for resistance to DDP has concurrently resulted in the loss of DNA mismatch repair function.

The loss of DNA mismatch repair in the 2008/A subline leads to the question of whether such loss itself contributes to the DDP-resistant phenotype. To address this, the cytotoxicity of DDP was compared in two pairs of cell lines replete or deficient in DNA mismatch repair and
isogenic except for the introduction of a chromosome carrying a copy of the functional gene. Parental HCT116 cells are DNA mismatch repair deficient due to lack of functional hMLH1. The DDP sensitivity of the HCT116+ch3-6 subline, into which a functional copy of hMLH1 had been introduced by transfer of a wild-type copy of chromosome 3, was compared to that of the HCT116+ch2-1 subline into which chromosome 2 had been inserted as a control. A similar comparison was made between HEC59 cells which lack a functional hMSH2 gene and a subline complemented with a wild-type copy of hMSH2 on chromosome 2 (HEC59+ch2–4). Table 2 shows that the hMLH1-deficient HCT116 cells were 2-fold resistant as compared to the proficient subline HCT116+ch3-6 (P < 0.05 in a two-sided unpaired t test). Similarly, the hMSH2-deficient HEC59 cells were 1.8-fold resistant as compared to the complemented subline HEC59+ch2-4 (P < 0.05 in a two-sided unpaired t test). Therefore, loss of DNA mismatch repair due to the deficiency of either hMLH1 or hMSH2 confers a similar degree of resistance to DDP.

DISCUSSION

The present study indicates that loss of DNA mismatch repair contributes to resistance in the absence of any prior exposure to DDP. Although the magnitude of the change in DDP sensitivity in the HCT116 and the HEC59 cells as compared to their mismatch repair competent-complemented clones (HCT116+ch3-6 and HEC59+ch2-4) was not large, it is important to note that changes of <2-fold in DDP sensitivity are sufficient to account for the clinical failure of this drug (32). In addition, the study demonstrates that selection of cells for resistance to DDP can result in the loss of hMLH1 expression, and that such loss of hMLH1 protein results in a defect in DNA mismatch repair.

The loss of hMLH1 in 2008/A human ovarian carcinoma cells was associated with most of the elements of the expected phenotype: the cells acquired resistance to the DNA-methylating agent MNNG; microsatellite instability was easily demonstrable; and the mutation rate at the HPRT locus was elevated. Although the observed increase in the mutation rate at the HPRT locus was lower than that reported earlier (12) in colon carcinoma cell lines, it was in close accordance with the results of other studies (31, 33). The only additional component of the phenotype anticipated on the basis of studies of the loss of hMLH1 function in other cell systems was resistance to 6-thioguanine. Hawn et al. (29) found that loss of hMLH1 in human HCT116 colon carcinoma cells was accompanied by the appearance of resistance to 6-thioguanine, and similar findings have been reported in other cell lines (15, 16). In contrast to these reports, the loss of hMLH1 expression in the 2008/A cells was accompanied by the appearance of mild hypersensitivity to 6-thioguanine. The basis for this is not apparent at the present time, but it is likely to be related to the specifics of the mutational changes that have occurred in these particular cells during the repeated selections with increasing doses of DDP. It is conceivable that the DDP selection resulted in changes that confer 6-thioguanine hypersensitivity that are not related to either loss of DNA mismatch repair function or alterations at the HPRT locus (34, 35). Even in the absence of a mechanistic understanding, this finding permits the conclusion that 6-thioguanine resistance is not a universal feature of the loss of hMLH1 expression or DNA mismatch repair.

Although selection of the 2008/A cells was accompanied by the loss of DNA mismatch repair, such loss is not a necessary concomitant of the emergence of DDP resistance: the 2008/C13*5.25 (20) and 2008/A (19) cells were selected using the same strategy of repeated in vitro exposure, and yet the 2008/C13*5.25 cells have normal expression of hMLH1 and hMSH2 with immunoblot. In addition, the absence of microsatellite instability in the 2008/C13*5.25 cells argues against the loss of expression of any other genes whose function is essential to DNA mismatch repair. The frequency of loss of DNA mismatch repair after exposure to cisplatin cannot be estimated from this study. However, microsatellite instability was described recently as a frequent occurrence after exposure of cells to DDP (36).

The extent to which loss of DNA mismatch repair contributes to DDP resistance in 2008/A cells is not entirely clear. There are indeed two possible ways in which the loss of DNA mismatch repair and DDP resistance in the 2008/A cells may be related to each other. The loss of DNA mismatch repair may not be the cause but rather be a secondary result of random DDP-induced mutagenesis that accompanies the emergence of some other molecular mechanism that confers DDP resistance. Alternatively, the loss of DNA mismatch repair might directly contribute to DDP resistance. This latter concept is supported by the finding of resistance to DDP in two unrelated cell lines that are deficient in hMLH1 (HCT116) and hMSH2 (HEC59), respectively. MSH2 protein can bind to platinated DNA, and there is ample circumstantial evidence for the binding of DNA mismatch repair proteins to O6-methylguanine in DNA following exposure to MNNG (30, 37–40) and to DNA containing 6-thioguanine (16, 31). The involvement of hMLH1 in a pathway controlling the G2-M checkpoint was recently described by Hawn et al. (29). It is, therefore, conceivable that the DNA mismatch repair system plays a central role in recognizing the presence of adducts in the DNA and generating the signal that eventually triggers cell death. The observation of DDP resistance in cells that are deficient in hMLH1 and hMSH2 and the reversion of resistance after complementation of these cells with functional copies of the respective genes lends support to the latter concept.

The finding that the development of resistance to DDP can be accompanied by loss of DNA mismatch repair and the observation that loss of DNA mismatch repair contributes to resistance to DDP have several far-reaching implications for the problem of acquired clinical resistance to this drug. First, DDP-induced loss of DNA mismatch repair is likely to directly produce resistance to methylyating and possibly alkylating chemotherapeutic agents (14, 17, 33). The full spectrum of drugs affected and the magnitude of the effect is not yet known. However, the current results indicate that DDP can induce

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<th>Table 1 Mutation rates in 2008 and in 2008/A human ovarian adenocarcinoma cells</th>
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<td>Mutation rate</td>
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<tr>
<td>a (Luria-Delbrück, Ref. 25)</td>
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<td>b (99% confidence intervals)</td>
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<td>p (Galton-Watson, Ref. 26)</td>
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<td>99% confidence intervals</td>
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<td>a Estimation according to the “method of the mean” of Luria and Delbrück (25).</td>
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<td>b P &lt; 0.01.</td>
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<td>c Estimation according to a modified Galton-Watson model (26); geometric mean of 10 parallel cultures.</td>
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<th>Table 2 Cytotoxicity of DDP in DNA mismatch repair-deficient cells (HCT116 + ch2–1 and HEC59) and the respective complemented sublines (HCT116 + ch3–6 and HEC59 + ch2–4)</th>
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<td>Cell line (protein affected)</td>
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<tr>
<td>HCT116 (hMLH1)</td>
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<td>95% CI</td>
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<td>HEC59 (hMSH2)</td>
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^b CI, confidence intervals; means and 95% of at least three independent experiments in triplicate (P < 0.05).
resistance at least to itself under circumstances where it causes loss of DNA mismatch repair. Second, loss of DNA mismatch repair dramatically increases the mutation rate in the affected cells, and this has the potential of indirectly increasing the rate of development of resistance to many other classes of drugs and increasing the clinical aggressiveness of tumors by contributing to the mutational changes that underlie resistance. Thus, the finding that DDP exposure can result in loss of DNA mismatch repair mandates the examination of tumors before treatment and again at the time that they demonstrate clinical resistance to DDP to assess the incidence of treatment-induced loss of DNA mismatch repair in the in vivo setting.

REFERENCES

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