Resistance to Cytotoxic Drugs in DNA Mismatch Repair-deficient Cells

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ABSTRACT

Loss of DNA mismatch repair is a common finding in many types of sporadic human cancers as well as in tumors arising in patients with hereditary nonpolyposis colon cancer. The effect of the loss of DNA mismatch repair activity on sensitivity to a panel of commonly used chemotherapeutic agents was tested using one pair of cell lines proficient or deficient in mismatch repair due to loss of hMSh2 function and another due to loss of hMLH1 function. 6-Thioguanine-deficient in mismatch repair due to loss of hMSH2 function and another due to loss of hMLH1 function. 6-Thioguanine-N'-nitro-N-nitrosoguanidine, to which these cells are known to be resistant, are included in the panel as controls. The results were concordant in both pairs of cells. Loss of either hMSH2 or hMLH1 function was associated with low level resistance to cisplatin, carboplatin, and etoposide, but there was no resistance to melphanal, perosfamide, 5-fluorouracil, doxorubicin, or paclitaxel. The results are consistent with the concept that the DNA mismatch repair proteins function as a detector for adducts produced by 6-thioguanine, N-methyl-N'-nitro-N-nitrosoguanidine, cisplatin, and carboplatin, and that they play a role in detecting the DNA damage produced by the binding of etoposide to topoisomerase II and propagating signals that contribute to activation of apoptosis.

INTRODUCTION

Loss of DNA mismatch repair is the genetic basis for the hereditary nonpolyposis colon cancer syndrome and is a common finding in a variety of sporadic human neoplasms (reviewed in Ref. 1). Cells that lack DNA mismatch repair are typically resistant to the methylating agent MNNG (2, 3) and the antimitabolite 6-thioguanine (4, 5), and loss of DNA mismatch repair also confers low-level resistance to cisplatin (6–8). In addition to intrinsic resistance to certain drugs, DNA mismatch repair-deficient cells have high mutation rates not only in noncoding microsatellite sequences but also in genes such as HPRT (9), APRT (10), and the locus controlling ouabain resistance (11). It is, therefore, conceivable that in a population of DNA mismatch repair-deficient cells, a certain proportion will be resistant to any drug due to the high spontaneous mutation rate.

Because DNA mismatch repair deficiency is common in numerous types of malignant tumors (1) and minor changes in drug sensitivity of tumor cells can affect the clinical outcome (12), we sought to investigate the pattern of resistance to a panel of clinically important drugs in two well-characterized pairs of DNA mismatch repair-proficient and -deficient cell lines.

MATERIALS AND METHODS

Cell Lines. The hMLH1-deficient human colorectal adenocarcinoma cell line HCT116 was obtained from the American Tissue Type Collection (ATCC CCL 247); HCT116 contains a hemizygous mutation in hMLH1 resulting in a truncated, nonfunctional protein (13, 14). Sublines complemented with chromosome 3 (HCT116 + Ch3) and chromosome 2 (HCT116 + Ch2) were obtained from Drs. C. R. Boland and M. Koi. The chromosome 3-complemented cells are competent in DNA mismatch repair (14, 15). Similarly, the human endometrial adenocarcinoma cell line IEC59 is mutated at different loci on both alleles of hMSH2 and is deficient in mismatch repair activity (13); a subline complemented with chromosome 2 (HEC59 + Ch2) was obtained from Drs. C. R. Boland and M. Koi and expresses wild-type hMSH2. Both cell lines were maintained in Iscove’s modified Dulbecco 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 genetin (G418: 400 μg/ml for HCT116 + Ch3 and 600 μg/ml for HEC59 + Ch2). The absence and presence of expression of hMLH1 in HCT116 and HCT116 + Ch3 as well as of hMSH2 in HEC59 and in HEC59 + Ch2 was verified by immunoblot analysis (data not shown).

Materials. Carboplat, paclitaxel, and cisplatin were gifts from Bristol Myers-Squibb Co. (Princeton, NJ). MNNG, 4

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4 The abbreviation used is: MNNG, N-methyl-N'-nitro-N-nitrosoguanidine.
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Thereafter, the cells were washed, and new drug-free medium was added. Colonies of at least 50 cells were scored visually (cisplatin, carboplatin, and MNNG) or 24 h (all other drugs).

Antonio, TX).

6-thioguanine, etoposide, doxorubicin, 5-fluorouracil, melphalan, and sulforhodamine B were purchased from Sigma Chemical Co. (Missouri, MO). Perfosfamide (4-hydroperoxycyclophosphamide) was purchased from Omicron Biochemicals (San Antonio, TX).

Cytotoxicity Assays. Clonogenic assays were performed by plating either 250 HCT116 or 300 HECS9 cells into 60-mm tissue culture dishes. After 24 h, appropriate amounts of drug were added to the dishes, and the cells were exposed for 1 h (cisplatin, carboplatin, and MNNG) or 24 h (all other drugs). Thereafter, the cells were washed, and new drug-free medium was added. Colonies of at least 50 cells were scored visually after 10 to 12 days. Each experiment was performed at least three times with triplicate cultures for each drug concentration. IC_{50} were estimated by log-linear interpolation at a relative plating efficiency of 0.5.

The sulforhodamine B assay was used to assess effects of drugs on growth rate (16, 17). However, this assay yielded unreliable results with the HCT116 cells; thus, studies were performed only with the HECS9 cells. The cells were seeded into 96-well plates at a density of 6000 cells/well in 100 µl of medium. After 24 h, appropriate amounts of drugs were added in a final volume of 100 µl of medium. Control plates were fixed as described below to estimate the cellular protein at time 0 (T0). After 72 h, growth was stopped by adding 50 µl of 50% (w/v) trichloroacetic acid, and cellular protein was stained with sulforhodamine B and was measured by spectrophotometry (16). The relative growth rate R was calculated as previously reported (17): if T ≥ T0, r = (T - T0)/(C - T0); if T < T0, r = (T - T0)/T0, where T is the absorbance 72 h after drug treatment, C is the absorbance at 72 h in control untreated wells, and T0 is the absorbance in control wells measured immediately before drug treatment. Each of at least three independent experiments for each drug was performed in triplicate. IC_{50} were estimated by linear interpolation at r = 0.5. All drugs except melphalan and MNNG were diluted directly in medium immediately before use. MNNG and paclitaxel were prepared in DMSO; the volume of DMSO was kept at <0.1% (w/v) at all drug concentrations and in the respective controls. Previous experiments (data not shown) have established that 0.1% DMSO does not affect the viability or growth of these cell lines. Melphalan was prepared fresh for each experiment by dissolving it first in 0.1 M HCl in ethanol and then diluting it into tissue culture medium.

Atomic Absorption Spectroscopy. For drug accumulation studies with cisplatin, IC_{50} of HCT116 + ch2 and HCT116 + ch3 cells were treated with 100 µm cisplatin for 60 min. The cells were lysed in 2 ml of 1 M NaOH, and 25 µl of the crude lysate were measured on a Perkin-Elmer flameless atomic absorption spectrophotometer. For DNA platination studies, the cells were treated the same way and then lysed in 600 µl of a lysis buffer containing 2.6 M NaCl, 0.3 M EDTA (pH 8.0), and 1% SDS. The lysate was extracted with equilibrated phenol/chloroform/isoamyl alcohol, and then the DNA was precipitated with ethanol. RNA was digested with RNase A, and the DNA was again precipitated with ethanol. After re-suspension in TRIS-EDTA buffer, the DNA was quantitated by spectrophotometry at 260 nm. The DNA was then digested in 1 M HCl at 75° for 2 h, and 25 µl of the hydrolysate were used for the

### Table 1: IC_{50} concentrations for cells deficient or proficient in DNA mismatch repair on the basis of loss of hMLH1 function determined by clonogenic assay

| Drug     | HCT116 + ch2 | HCT116 + ch3 | P
|----------|--------------|--------------|--
| MNNG (µM) | 3.6^b        | 1.5^b        | 0.017
| 6-Thioguanine (µM) | >7.5^c | 1.8^c | 0.029
| Cisplatin (µM) | 24.3 ± 3.2^a | 11.4 ± 3.9 | 0.002
| Carboplatin (µM) | 125 ± 12 | 98 ± 7.4 | 0.029
| Perfosfamide (µM) | 1.83 ± 0.14 | 1.74 ± 0.38 | 0.71
| Melphalan (µM) | 7.6 ± 2.7 | 6.8 ± 2.0 | 0.71
| Flurouracil (µM) | 18.6 ± 5.4 | 15.8 ± 7.8 | 0.58
| Doxorubicin (nm) | 47.0 ± 38.2 | 28.7 ± 16.7 | 0.27
| Etoposide (µM) | 0.73 ± 0.27 | 0.36 ± 0.17 | 0.017
| Paclitaxel (nm) | 5.63 ± 1.38 | 3.67 ± 0.65 | 0.15

^a Two-sided t test.

^b Approximate value from Koi et al. (15).

^c Approximate value from Hawn et al. (18).

^d Average ± SD, n = 3.
The human endometrial adenocarcinoma cell line HEC59 lacks functional hMSSH2 (13). When compared with the chromosome 2-complemented subline HEC59 + ch2, it showed the typical phenotype of moderate resistance to the methylating agent MNNG and a high degree of resistance to 6-thioguanine (Fig. 2). Fig. 3 shows that the pattern of resistance in the hMSSH2-deficient HEC59 cells mimics that in the hMLH1-deficient colon HCT116 + ch2 cells, with the DNA mismatch repair-deficient cells demonstrating a moderate degree of resistance to cisplatin, carboplatin, and etoposide but no resistance to the alkylating agents or the other drugs tested (Table 2).

The pattern of resistance in the DNA mismatch repair-deficient cells was confirmed using the sulforhodamine B assay and the HEC59 and HEC59 + ch2 cells for a subset of the drugs tested by clonogenic assay. The data presented in Table 3 indicates that the two types of assays yielded largely concordant results, with the mismatch repair-deficient cells being more resistant to cisplatin, carboplatin, and etoposide. Again, melphalan inhibited the growth of both cell lines to a similar degree in the sulforhodamine B assay.

Impaired cellular accumulation of cisplatin is a common mechanism of resistance in the majority of cell lines selected for resistance to this drug (19). To exclude the possibility that resistance to cisplatin in the DNA mismatch repair-deficient cells was due to reduced uptake, total cellular accumulation and the extent of DNA platination was measured in cells exposed to 100 μM cisplatin for 1 h. The accumulation was similar in mismatch repair-deficient HCT116 + ch2 and proficient HCT116 + ch3 cells (303 ± 76 versus 289 ± 82 pmol/mg protein, P = 0.75). Likewise, the extent of total DNA platination in the HCT116 + ch2 cells was not significantly different from that in the HCT116 + ch3 cells (55.4 ± 22.4 versus 59.1 ± 17.4 fmol/μg DNA).

DISCUSSION

DNA mismatch repair is essential for the maintenance of genomic integrity. Cells that lack mismatch repair have increased mutation rates in both noncoding and coding sequences, which is manifest as microsatellite instability (reviewed in Ref. 1) and gene inactivation (9, 10, 20). In addition, mismatch...
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Table 2  IC50 concentrations for cells deficient or proficient in DNA mismatch repair on the basis of loss of hMSH2 function determined by clonogenic assay

<table>
<thead>
<tr>
<th>Drug</th>
<th>HEC59</th>
<th>HEC59 + ch2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNNG (μM)</td>
<td>12.5 ± 2.1</td>
<td>5.6 ± 1.5</td>
<td>0.001</td>
</tr>
<tr>
<td>6-Thioguanine (μM)</td>
<td>&gt;20</td>
<td>2.02 ± 1.23</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cisplatin (μM)</td>
<td>14.0 ± 1.2</td>
<td>7.9 ± 1.2</td>
<td>0.003</td>
</tr>
<tr>
<td>Carboplatin (μM)</td>
<td>51.6 ± 4.0</td>
<td>35.0 ± 4.3</td>
<td>0.008</td>
</tr>
<tr>
<td>Perfosfamide (μM)</td>
<td>2.07 ± 0.17</td>
<td>2.32 ± 0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Melphalan (μM)</td>
<td>2.73 ± 0.42</td>
<td>2.65 ± 0.38</td>
<td>0.82</td>
</tr>
<tr>
<td>Fluorouracil (μM)</td>
<td>47.4 ± 13.2</td>
<td>30.2 ± 6.0</td>
<td>0.11</td>
</tr>
<tr>
<td>Doxorubicin (nM)</td>
<td>22.4 ± 3.8</td>
<td>20.1 ± 4.3</td>
<td>0.46</td>
</tr>
<tr>
<td>Etoposide (μM)</td>
<td>1.18 ± 0.29</td>
<td>0.66 ± 0.07</td>
<td>0.013</td>
</tr>
<tr>
<td>Paclitaxel (nM)</td>
<td>1.12 ± 0.39</td>
<td>0.96 ± 0.43</td>
<td>0.66</td>
</tr>
</tbody>
</table>

* Two-sided t test.
* Average ± SD, n = 3.

Table 3  IC50 concentrations for cells deficient or proficient in DNA mismatch repair on the basis of loss of hMSH2 function determined by sulforhodamine B assay

<table>
<thead>
<tr>
<th>Drug</th>
<th>HEC59</th>
<th>HEC59 + ch2-4</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin (μM)</td>
<td>13.4 ± 1.56</td>
<td>7.5 ± 1.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Carboplatin (μM)</td>
<td>139 ± 21</td>
<td>77 ± 22</td>
<td>0.007</td>
</tr>
<tr>
<td>Melphalan (μM)</td>
<td>31.9 ± 0.95</td>
<td>32.2 ± 5.15</td>
<td>0.93</td>
</tr>
<tr>
<td>Fluorouracil (μM)</td>
<td>17.4 ± 3.6</td>
<td>14.9 ± 2.1</td>
<td>0.35</td>
</tr>
<tr>
<td>Etoposide (μM)</td>
<td>11.8 ± 1.02</td>
<td>6.9 ± 2.73</td>
<td>0.01</td>
</tr>
<tr>
<td>Paclitaxel (nM)</td>
<td>1.60 ± 0.38</td>
<td>1.72 ± 0.32</td>
<td>0.70</td>
</tr>
</tbody>
</table>

* Two-sided t test.
* Average ± SD, n = 3.

repair-deficient cells are resistant to MNNG and 6-thioguanine (2–5). Recently, we and others have demonstrated that loss of hMLH1 and hMSH2 also confers low level resistance to cisplatin (6–8) and carboplatin (21). Temozolomide, one of the few methylaing agents in clinical use, forms the same adducts in DNA as MNNG, and mismatch repair-deficient cells have recently been reported to be resistant to this drug (22, 23). Defective DNA mismatch repair is common in some types of sporadic cancers and is the molecular basis for the hereditary portions of each type of adduct differ between the two agents. Identical types of adducts in DNA, although the relative proportions of each type of adduct differ between the two agents. Because loss of mismatch repair is not accompanied by resistance to other platinum compounds such as oxaliplatin (21) and to the classical alkylators melphalan and perfosfamide, at least over the first log of cell kill, it is likely that the adducts produced by these agents are not recognized by the mismatch repair detector function. The lack of differential cytotoxicity of 5-fluorouracil and paclitaxel was anticipated based on the fact that cisplatin and carboplatin produce identical types of adducts in DNA, although the relative proportions of each type of adduct differ between the two agents. Because loss of mismatch repair is not accompanied by resistance to other platinum compounds such as oxaliplatin (21) and to the classical alkylators melphalan and perfosfamide, at least over the first log of cell kill, it is likely that the adducts produced by these agents are not recognized by the mismatch repair detector function. The lack of differential cytotoxicity of 5-fluorouracil and paclitaxel was anticipated based on the fact that the small amounts of 5-fluorouracil that are incorporated into DNA are removed efficiently by uracil glycosylase, and the fact that paclitaxel is not known to interact with DNA. The challenge now will be to determine whether all elements of the DNA mismatch repair complex are required for detector function. In this regard, we can speculate that the binding of the hMSH2-GTBP or hMSH2-hMSH3 dimers alone is not sufficient for detector function because cells containing normal amounts of hMSH2 but lacking hMLH1 (HCT116 + ch2) were still resistant to cisplatin. This is consistent with the hypothesis that assembly on the damaged DNA of at least either hMSH2-GTBP or hMSH2-hMSH3 and the hMLH1-hPMS2 heterodimer are required before a damage signal can be generated. The finding that cells deficient in DNA mismatch repair were resistant to etoposide is of particular interest. This observation is consistent with the hypothesis that the DNA mismatch repair proteins are involved in detecting the “cleavable complex” generated by the binding of etoposide to topoisomerase II (27). Both hMSH2 and hMLH1 would appear to be required for after a 1-h exposure to cisplatin. Thus, the difference in sensitivity to cisplatin cannot be accounted for on the basis of differential drug uptake or differential detoxification of cisplatin in the cytosol prior to its reaction with DNA. It has been suggested that the DNA mismatch repair proteins serve as a detector system for the presence of DNA damage (2, 18, 25). Indeed, human hMutSα, a heterodimer of hMSH2 and GTBP, has been shown to bind to DNA containing adducts produced by MNNG, 6-thioguanine, and cisplatin (25), and pure hMSH2 has been reported to bind to platinated DNA in gel shift assays (26). The paradigm is that loss of the detector function of the DNA mismatch repair system results in failure to generate signals that contribute to the activation of the apoptotic response; therefore, these cells are resistant to the cytotoxic effect of these agents. The observation that loss of DNA mismatch repair is associated with resistance to carboplatin is consistent with the fact that cisplatin and carboplatin produce identical types of adducts in DNA, although the relative proportions of each type of adduct differ between the two agents. Because loss of mismatch repair is not accompanied by resistance to other platinum compounds such as oxaliplatin (21) and to the classical alkylators melphalan and perfosfamide at least over the first log of cell kill, it is likely that the adducts produced by these agents are not recognized by the mismatch repair detector function. The lack of differential cytotoxicity of 5-fluorouracil and paclitaxel was anticipated based on the fact that the small amounts of 5-fluorouracil that are incorporated into DNA are removed efficiently by uracil glycosylase, and the fact that paclitaxel is not known to interact with DNA at all.

The challenge now will be to determine whether all elements of the DNA mismatch repair complex are required for detector function. In this regard, we can speculate that the binding of the hMSH2-GTBP or hMSH2-hMSH3 dimers alone is not sufficient for detector function because cells containing normal amounts of hMSH2 but lacking hMLH1 (HCT116 + ch2) were still resistant to cisplatin. This is consistent with the hypothesis that assembly on the damaged DNA of at least either hMSH2-GTBP or hMSH2-hMSH3 and the hMLH1-hPMS2 heterodimer are required before a damage signal can be generated. The finding that cells deficient in DNA mismatch repair were resistant to etoposide is of particular interest. This observation is consistent with the hypothesis that the DNA mismatch repair proteins are involved in detecting the “cleavable complex” generated by the binding of etoposide to topoisomerase II (27). Both hMSH2 and hMLH1 would appear to be required for
this detection process because etoposide resistance was observed in the HEC59 and HCT116 cell models. It should be noted that doxorubicin resistance has been reported in sublines of the human ovarian carcinoma cell line A2780 found to have deficient MutLa activity after in vitro selection for resistance to cisplatin or doxorubicin (8). Additional studies will be required to document interaction between the DNA mismatch repair proteins and the topoisomerases.

What is the likely clinical significance of the relatively low level resistance conferred by loss of DNA mismatch repair? In the case of cisplatin, less than a 2-fold reduction in sensitivity is sufficient to account for in vivo failure of treatment with this agent (12). This has been confirmed recently by xenotransplantation studies of MSH2 knock-out and proficient embryonic stem cells in athymic nude mice, where we observed significant resistance of the MSH2-deficient tumors to cisplatin (28). In the case of etoposide, the contribution of low level resistance to clinical failure is unknown, but such resistance would be expected to result in the gradual enrichment for genomically unstable mismatch repair-deficient cells during a course of treatment. This can be anticipated to be therapeutically unfavorable because these cells have high mutation rates at multiple loci, including those that control sensitivity to other classes of agents.

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