Hypersensitivity of Tumor Cell Lines with Microsatellite Instability to DNA Double
Strand Break Producing Chemotherapeutic Agent Bleomycin

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ABSTRACT

Genetic or epigenetic inactivation of DNA mismatch repair genes results in a strong mutator phenotype, known as the microsatellite mutator phenotype or microsatellite instability (MSI). This mutator phenotype causes mutations in genes responsible for the regulation of cell growth and survival/death and thus promotes the development and progression of tumors. In addition to such tumorigenic lesions, mutations in genes of other types of DNA repair, for example, DNA double-strand break (DNA DSB) repair, are found in tumor cells with MSI. We report here that the majority of MSI-positive tumor cell lines of different tissue origins (endometrial, ovarian, prostate, and colorectal carcinomas) are hypersensitive to bleomycin, a DNA DSB producing chemotherapeutic drug. We suggest that this hypersensitivity may be a result of inactivation of the DNA DSB repair activity by concomitant mutations of different DNA DSB repair genes. To provide experimental support to this hypothesis, we show that the subclones of the MSI-positive colorectal cancer cell line HCT-8 that bear heterozygous frameshift mutations in the DNA DSB repair gene DNA-PKcs are more sensitive to a combined treatment with bleomycin and the DNA protein kinase inhibitor LY294002 than the original HCT-8 cells, which are wild type for this gene. These results may be useful in designing therapies for MSI-positive cancer.

INTRODUCTION

DNA mismatch repair (DNA MMR) plays a crucial role in the maintenance of genomic stability during DNA replication by correcting nucleotide mismatches and small DNA strand loops that escape DNA polymerase proofreading. Several homologues of bacterial NER genes, hMSH2, hMSH3, hMSH5, hMSH6, hMLH1, hMLH3, hPMS1, and hPMS2, have been identified in humans (1). Although a general model of mammalian MMR has been proposed, the exact role of each of the DNA MMR homologues in DNA repair is not completely understood.

Loss of DNA MMR function is a landmark of hereditary nonpolyposis colorectal cancer and a minority of sporadic cancers of various tissue origins. Because of the inability of the DNA MMR-defective cells to correct replication errors, hundreds of thousands of mutations accumulate throughout the cell genome. This mutator phenotype was first discovered by the ubiquitous presence of slippage-induced insertion/deletion mutations in microsatellite repeat sequences, for which reason it is often called microsatellite mutator instability (MSI) (2, 3). Besides the multitude of mutations in non-functional microsatellite sequences, other mutations also occur in relevant genomic DNA sequences, ultimately leading to neoplastic transformation and tumor progression (4). The genes that contain mononucleotide repeats or microsatellites are especially prone to mutations in cells with compromised MMR because of the intrinsic instability of these sequences. Preferential accumulation of mutations in the repeat-containing genes that are implicated in cell growth and differentiation, cell motility, cell cycle control, and cell death, followed by sequential selection of clones that exhibit neoplastic properties, leads to the tumor progression of MSI-positive cancers (5, 6).

In addition to predisposing tumorigenesis, loss of MMR function is associated with an altered sensitivity of tumor cells to a variety of commonly used chemotherapeutic drugs. MMR-deficient tumor cells exhibit elevated resistance to antitumor alkylating agents (busulfan, procarbazine, methylnitrosourea, methylnitrosoguanidine, and temozolomide), platinum compounds (cisplatin and carboplatin), topoisomerase poisons (doxorubicin, epirubicin, etoposide, and mitoxantrone), and purine analogs (mercaptopurine and 6-thioguanine) (7). The mechanism of this drug resistance is presumably linked to the inability of MMR-deficient cells to recognize specific types of DNA damage and promote cell apoptotic death because of either inactivation of the MMR genes themselves (8–11) or inactivation of proapoptotic genes, such as BAX, APAF-1, Bcl-10, and FAS (5, 12, 13). It is important, therefore, to identify anticancer agents that retain activity against MMR-deficient tumors.

A number of studies showed that MSI-positive tumors bear inactivating mutations in mononucleotide repeats of the MRE11, RAD50, BRCAl, BRCa2, BLM, and ATM genes, the protein products of which are involved in the repair of DNA double strand breaks (DNA DSB; Ref. 14–17). We hypothesized that these defects of DNA DSB repair may sensitize MSI-positive tumor cells to DNA DSB producing agents, such as ionizing radiation and radiomimetic drugs methyl methanesulfonate or bleomycin. In the present study, we showed that, in contrast to MSI-negative tumor cell lines, MSI-positive tumor cell lines of different tissue origins are hypersensitive to bleomycin, a clinically used antitumor agent that produces DNA DSB.

MATERIALS AND METHODS

Tumor Samples, Cell Lines, and Media. Gastric and colon tumor and DNA specimens were obtained from the Cooperative Human Tissue Network and Oncomatrix, Inc. (San Diego, CA). The human tumor cell lines SK-UT-1B (uterine mixed mesodermal tumor), AN3CA (endometrial carcinoma), DU145 (prostate carcinoma), and HCT-8, LS180, DLD-1, HCT116, LoVo, SW48, WiDr, SW1417, SW1116, LS123, SW620, LS411N (colorectal carcinomas) were obtained from the American Type Culture Collection. The cells were grown in DMEM medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (Tissue Culture Biological, Tulare, CA), 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Inc., Gaithersburg, MD).

Cell Survival Assays. Survival curves for the cell lines were obtained by colony-forming assays. The cells were treated with bleomycin (Sigma, St. Louis, MO) or LY294002 (2-[4-(morpholino)-8-phenyl-4H-1-benzopyran-4-one] [Calbiochem, San Diego, CA) alone or in combination, grown for 14 days, fixed and stained with 1% crystal violet in a 70% ethanol solution. Colonies containing >50 cells were scored. Each experiment was performed three times for each drug concentration. The exponential approximation of the scatter plots (one phase exponential decay, \( Y = ae^{-bx} + c \)) was done with the Prism 2.0 software (GraphPad Software, San Diego, CA) to determine the bleomycin concentrations producing 70% inhibition of cell growth (IC \(_{70}\)).

Msi Status and p53 Gene Mutations. The MSI status of the tumors and the LS123 colon carcinoma cell line was determined by the PCR amplification of two mononucleotide repeats, BAT26 (18) and ApΔ3 (4). The MSI status of...
The rest of the cell lines has been reported previously (17, 19–21). Genetic alterations of the hMLH1, hMSH2, hMSH6, and p53 genes in the cell lines are summarized in Table 1.

### Analysis of Mutations within the Simple Repeats of the DNA DSB Repair Genes

Genomic DNA from the cell lines and primary tumors was extracted by a standard phenol-chloroform method. Deletion/insertion mutations within the mononucleotide repeats of the BLM, ATR, DNA-PKcs, BRC1, BRC2, RAD50, and MRE11 genes were detected by PCR followed by denaturing gel electrophoresis. The PCR was performed in a 10 μl final volume containing 40 ng of genomic DNA, 1 unit of Jump-start Taq polymerase, 1× TaqPCR buffer, 2.5 mm MgCl2, 125 μM of each deoxynucleotide triphosphate (Sigma), 2 μCi of [α-32P]dCTP (Perkin-Elmer, Boston, MA) and 0.5 μM of each primer. The PCR primers were as follows: 5'-TTGTAAGGCACTGAGGCTG-3', forward, and 5'-TCATCTCATCAAGTCTGTC-3', reverse, for the BLM gene (polyA) repeat; 5'-CCGGCAGCTTATGAGGCTG-3', forward, and 5'-TAAGGACCCTGGTAGAAGAAAAC-3', reverse, for the DNA-PKcs gene (polyA) repeat; 5'-TGACAGAGTTGGAGCAATGATG-3', forward, and 5'-GCCAGTCCATACCTGTAATAA-3', reverse, for the DNA-PKcs gene poly(A) repeat; 5'-CTAAACGCGGACTTGCTTCA-3', forward, and 5'-TCTTAACCTATGGCGACAAG-3', reverse, for the RAD50 gene poly(A) repeat; 5'-CTTGGAGAAACAATTTGGG-3', forward, and 5'-TTGGAATACAGATTTGTAAG-3', reverse, for the RAD50 gene poly(A) repeat; 5'-TTGGAGAAACAATTTGGG-3', forward, and 5'-ATTAGGAAATGGTTGTTG-3', reverse, for the MRE11 gene (polyA) repeat; 5'-TTGAGCTTATCCTGATCTG-3', forward, and 5'-ATTAGGAAATGGTTGTTG-3', reverse, for the MRE11 gene (polyA) repeat; 5'-ATGGAGCTTATCCTGATCTG-3', forward, and 5'-ATTAGGAAATGGTTGTTG-3', reverse, for the RAD50 gene exon 2 poly(A) repeat.

Bands were visualized by autoradiography.

### Results

#### Sensitivity of Tumor Cell Lines to Bleomycin

We applied two different regimes of bleomycin treatment to cell cultures. In the first regime of bleomycin treatment, the majority of the MSI-positive cell lines survived worse than the MSI-negative ones (Fig. 1A). Whereas the survival rates of seven of the nine MSI-positive cell lines were below 17% at the maximum dose of bleomycin, the survival rates of four out the five MSI-negative cell lines were higher than 50%. Among MSI-positive cell lines, DLD-1 and HCT-8 were less sensitive to bleomycin: e.g., at 40 μg/ml bleomycin their survival was 53% and 39%, respectively. LS123, an MSI-negative cell line, exhibited an increased sensitivity to bleomycin and had 21% survival at the maximum dose of the drug.

In the second treatment regime, 8 of 10 MSI-positive cell lines exhibited high sensitivity to bleomycin. Their survival varied from as low as 1% to 7% at the maximum drug dose, whereas the survival rates of four of five MSI-negative cell lines ranged between 36% and 53% (Fig. 1B). Again, the survival curves of the three cell lines, DLD-1, HCT-8, and LS123, lay outside of the overall trend exhibited by the other cell lines with the same MSI status. At the maximum dose of bleomycin, the survival rate of the DLD-1 and HCT-8-MSI-positive cell lines was 34% and 60%, respectively, which is significantly higher than the average survival for the rest of the MSI-positive cell lines. On the other hand, the survival rate of the LS123 MSI-negative cell line was 3%, significantly lower compared with the other MSI-negative cell lines.

We evaluated the sensitivity of the cell lines to bleomycin by calculating the drug concentration that corresponds to a 70% survival rate (IC70). The reason for using IC70 instead of more commonly used 50% inhibition concentration (IC50) was that the survival varied greatly between the MSI-positive and -negative cell lines, whereas the survival of some MSI-positive cell lines at the maximum dose of the drug was <1%, the survival of MSI-negative cell lines was >50%. DLD-1 and HCT-8 had the highest IC70 among the MSI-positive cell lines: e.g., 10.01 and 12.31 μg/ml, respectively, in the first regime and 0.64 and 0.74 μg/ml, respectively, in the second regime of bleomycin treatment. IC70 for the rest of the cell lines was considerably lower for the MSI-positive cell lines compared with MSI-negative: e.g., in the first regime of bleomycin treatment, IC70 ranged between 0.78 and 3.19 μg/ml versus 8.30 and 41.67 μg/ml, respectively (Fig. 2A), and in the second regime between 0.04 and 0.26 μg/ml versus 0.28 and 1.35 μg/ml, respectively (Fig. 2B).

Thus, there is a clear trend toward a higher sensitivity of the MSI-positive cell lines to bleomycin. With the noted exceptions of the DLD-1 and HCT-8-MSI-positive and the LS123 MSI-negative cell lines, the average survival rate of MSI-positive cells was about 1 order of magnitude lower than MSI-negative cells. Accordingly, the average bleomycin IC70 value was about 1 order of magnitude lower for MSI-positive cells compared with MSI-negative.

### Mutational Status of the p53 and DNA DSB Repair Genes in Tumor Cell Lines and Primary MSI-Positive Tumors

To test the hypothesis that the hypersensitivity of the MSI-positive cells to bleomycin is associated with inactivation of the DNA DSB repair system, we analyzed the mononucleotide repeats within the BLM, ATR, DNA-PKcs, BRC1, BRC2, RAD50, and MRE11 genes for the presence of mutations in the cell lines and primary tumors with MSI. We also examined the cell lines (by search of published data) and MSI-positive primary tumors (by experimental analysis) for the presence of mutations in the entire coding region of the p53 gene, which is involved in recognition and repair of many types of DNA lesions, including DNA DSB.

The majority of the MSI-positive cell lines had concomitant mutations in the mononucleotide repeats of different DNA DSB repair genes (Fig. 3; Table 2). The LS411N cells carried homozygous mutations in the MRE11 gene and heterozygous mutations in the BML, ATR, DNA-PKcs, and RAD50 genes. The LoVo cells carried three homozygously mutated genes, BLM, RAD50, and MRE11. The
SK-UT-1B and DU145 cell lines each carried three heterozygously mutated genes, ATR, RAD50, MRE11, and DNA-PK CS, RAD50, MRE11, respectively. The LS180 cell line carried a homozygously mutated MRE11 and a heterozygously mutated BRCA2 and RAD50. The HCT116 and AN3CA cell lines each carried a homozygously mutated MRE11 and a heterozygously mutated RAD50. The HCT-8 and DLD-1 cell lines each carried only a heterozygously mutated MRE11. No frameshift mutations were found within the poly(A) tract of the BRCA1 gene and the poly(A) tract of the BRCA2 gene exon 9 in any of the cell lines. No mutations were found within the simple repeats of the studied genes in the LS123 cell line. The data on the p53 gene mutations for the cell lines are shown in Table 1. The MSI-negative cell lines were more frequently mutated for p53 than the MSI-positive cell lines (three of the three analyzed versus two of the eight analyzed, respectively).

A meta-analysis together with our data on the frequency of mutations within the mononucleotide tracts of the BLM, ATR, DNA-PK CS, BRCA1, BRCA2, RAD50, and MRE11 genes and the entire coding region of the p53 gene in MSI-positive primary colon and stomach tumors is shown in Table 3. Besides the high frequency of mutations in each gene in MSI-positive tumor cell lines, our analysis also showed that about 75% of colon tumors contained either a homozygous mutation in one of the genes (mostly MRE11) or concomitant heterozygous mutations in two or more different DNA DSB repair genes. About 55% of colon tumors contained either a homozygous mutation in one of the genes or three or more coexisting heterozygous mutations in different genes (data not shown).

Thus, MSI-positive tumors and tumor cell lines are characterized by a high frequency of mutations in the DNA DSB repair genes, whereas the MSI-negative phenotype is associated with a higher frequency of p53 gene mutations.

Sensitivity to Bleomycin of HCT-8 Cell Line and HCT-8 Derivative Cell Lines with Mutations in the DNA-PK CS Gene. As described previously, HCT-8 is more resistant to bleomycin compared with the other MSI-positive cell lines. The HCT-8 cells carry only a heterozygously mutated MRE11 gene and, according with our hypothesis, should have less compromised DNA DSB repair activity. To further test the hypothesis that mutations in DNA DSB repair genes affect the cell sensitivity to bleomycin, we compared bleomycin sensitivity between the HCT-8 cell line and its derivatives with additional mutations in the DNA-PK CS gene. To obtain DNA-PK CS mutant cells, we used a subcloning approach that was developed previously in our laboratory (39). Because mononucleotide repeats are preferential targets for MSI, deletion/insertion mutations occur with high frequency in such sequences during cell passages in vitro. Thus, cells mutated for a particular mononucleotide sequence can be isolated by subcloning and analyzing a sufficient number of clones for the presence of mutations. Two independent clones that carry a single-nucleotide deletion mutation in the poly(A) repeat of one of the two alleles of the DNA-PK CS gene (heterozygous mutations) were isolated by this procedure (Fig. 4). The
survival rate was compared between these cell lines and the original HCT-8 cells under different conditions of bleomycin treatment. Treatment with bleomycin alone resulted in no difference in survival between the HCT-8 wild-type and HCT-8/DNA-PK CS\(^{-}\)/H11006 mutant cells (data not shown). This can be explained by the assumption that, under the experimental conditions, the transcription from a single wild-type DNA-PK CS allele in HCT-8/DNA-PK CS\(^{-}\)/H11006 mutant cells provided a sufficient amount of cellular DNA-dependent protein kinase catalytic subunit (DNA-PKCS) to repair DNA DSB with efficiency equal to the HCT-8 cells. We hypothesized, however, that further inhibition of the cellular DNA-protein kinase (DNA-PK) activity should increase bleomycin sensitivity more efficiently in the case of heterozygous mutant cells than in the wild-type cells.

To test this hypothesis, we treated cells with a high dose of bleomycin to maintain a constant level of DNA DSB formation and, at the same time, applied increasing concentrations of LY294002, a DNA-PK inhibitor that interacts with the catalytic phosphatidyl 3-kinase domain of the DNA-PK (40). Under these experimental conditions, the survival of the mutant cells was lower compared with the wild-type cells (Fig. 5). Employment of two independent subclone cell lines with the DNA-PK CS\(^{-}\)/H11006 genotype ensured that the differences in the bleomycin sensitivity between the mutant and wild-type cells was not related to genetic alterations other than the DNA-PK CS\(^{-}\) mutation. The probability that a different mutation(s) was responsible for the effect in two independent mutant cell lines can be estimated to be extremely low.

**DISCUSSION**

In this study, we showed that the MSI phenotype or, respectively, the DNA MMR deficiency, is associated with the hypersensitivity of tumor cells to the chemotherapeutic agent bleomycin. Bleomycin is a clinically used antitumor antibiotic that produces DNA DSB and single strand breaks. It preferably cuts DNA within actively transcribed domains, depending on the accessibility of the chromatin for cleavage (41). The cytotoxicity of bleomycin is predominantly attributed to the DNA DSB production because DNA DSB are the most dangerous type of DNA damage. By comparison of the cytotoxic concentrations of bleomycin and deglyco-bleomycin (a bleomycin derivative that generates almost exclusively single strand breaks), it was shown that DSB have 300 times higher cellular toxicity than single strand breaks (42).

We ruled out a hypothesis that inactivation of the DNA MMR genes can be the primary cause for the observed sensitivity of MSI-positive cells to bleomycin. It was shown previously that restoring of DNA MMR function by introducing either a wild-type DNA MMR gene or demethylating the hMLH1 gene do not change the sensitivity of cells to another DSB producing agent \(\gamma\)-radiation (43, 44). Instead, we propose that the hypersensitivity results from a partial or complete inactivation of the cellular DNA DSB repair caused by accumulation of heterozygous/homozygous mutations in DNA DSB repair genes during many cell divisions of DNA MMR-deficient tumor precursor cells.

Eukaryotic cells have the following two distinct mechanisms of DNA DSB repair: homologous recombination and non-homologous end joining (45). DNA-PK, which consists of the catalytic subunit and the regulatory complex of Ku70 and Ku80 proteins, XRCC4 and DNA ligase IV are critical for the repair of DNA ends by non-homologous end joining (46). The proteins of the RAD family (RAD50, RAD51, and RAD54), RAD-related proteins (XRCC2, XRCC3, RAD51B, RAD51C, and RAD51D), c-Abl tyrosine kinase, MRE11, NBS1 (45), and BRCA2 (47) participate in the homologous recombination pathway. The two pathways of the DNA DSB repair are partially complementary and require interaction with ATM, BLM, BRCA1 (48), ATR (49) and p53 for the primary recognition of the DNA damage.

We showed that the vast majority of MSI-positive cell lines and...
primary tumors of different tissue origins have hetero- and homozygous mutations in one or several DNA DSB repair genes, BLM, ATR, DNA-PK, BRCAl, BRCAl2, RAD50, and MREII1 (Tables 2 and 3). We confirmed the existence of a heterozygous deletion mutation within the ATR gene poly(A) tract reported previously for the SK-UT-1B cell line (50), as well as mutations in the mononucleotide tract of BLM (53) knockout mice and in rodent cell lines with the lower frameshift mutation frequency in DLD-1 and HCT-8 cells is explained by the presence in each cell line of only one identical heterozygous mutation. Although this does not exclude a possibility that these cell lines may not sufficiently reduce the DNA DSB repair activity. Mononucleotide repeats in six other repair genes were not mutated in these cell lines. Although this does not exclude a possibility that these cell lines carry mutations in the other regions of the DNA DSB repair genes that were analyzed, or in genes that were outside the scope of this study, the lower frameshift mutation frequency in DLD-1 and HCT-8 cells is in agreement with the mild mutator phenotype of these cells (19, 30).

### Table 2. Mutations in mononucleotide repeats of DNA DSB repair genes in cell lines

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence examined for mutations</th>
<th>Current study</th>
<th>Meta-analysis</th>
</tr>
</thead>
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<tr>
<td>BLM</td>
<td>(A)₉</td>
<td>13% [7/54]</td>
<td>13%</td>
</tr>
<tr>
<td>ATR</td>
<td>(A)₁₀</td>
<td>24% [11/46]</td>
<td>18%</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>(A)₉₋₁₀ (A)₉</td>
<td>41% [20/49]</td>
<td>nd</td>
</tr>
<tr>
<td>BRCAl</td>
<td>(A)₈</td>
<td>4% [2/55]</td>
<td>2%</td>
</tr>
<tr>
<td>BRCAl2</td>
<td>(A)₈</td>
<td>7% [4/55]</td>
<td>1%</td>
</tr>
<tr>
<td>RAD50</td>
<td>(A)₁₀</td>
<td>41% [23/56]</td>
<td>26%</td>
</tr>
<tr>
<td>MREII1</td>
<td>(T)₁₁</td>
<td>68% [25/41]</td>
<td>93%</td>
</tr>
<tr>
<td>p53</td>
<td>Entire coding region</td>
<td>26% [17/66]</td>
<td>nd</td>
</tr>
</tbody>
</table>

### Table 3. Frequency of the p53 and the DNA DSB repair gene mutations in primary MSI-positive tumors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence examined for mutations</th>
<th>Colon cancer</th>
<th>Stomach cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLM</td>
<td>(A)₉</td>
<td>13% [7/54]</td>
<td>13%</td>
</tr>
<tr>
<td>ATR</td>
<td>(A)₁₀</td>
<td>24% [11/46]</td>
<td>18%</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>(A)₉₋₁₀ (A)₉</td>
<td>41% [20/49]</td>
<td>nd</td>
</tr>
<tr>
<td>BRCAl</td>
<td>(A)₈</td>
<td>4% [2/55]</td>
<td>2%</td>
</tr>
<tr>
<td>BRCAl2</td>
<td>(A)₈</td>
<td>7% [4/55]</td>
<td>1%</td>
</tr>
<tr>
<td>RAD50</td>
<td>(A)₁₀</td>
<td>41% [23/56]</td>
<td>26%</td>
</tr>
<tr>
<td>MREII1</td>
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<td>p53</td>
<td>Entire coding region</td>
<td>26% [17/66]</td>
<td>nd</td>
</tr>
</tbody>
</table>

The poly(A)₉ tract within the exon 9 of the BRCAl2 gene.
It is highly probable that DLD-1 and HCT-8 are derived from the same patient. Although DLD-1 but not HCT-8 carries a C722T mutation in one of the two alleles of the p53 gene (Ref. 19, 35, and 37; Fig. 6A), both cell lines have identical DNA fingerprint profiles (58) and, in addition to the identical MRE11 mutation, harbor the same 5bp deletion mutation (tgatagag→tttgt) at the exon 6 of the hMSH6 gene (Fig. 6A), which was reported previously for DLD-1 (36). Presumably, DLD-1 was derived from HCT-8, but acquired the p53 gene mutation during in vivo maintenance.

The DLD-1 and HCT-8 colon carcinoma cell lines harbor biallelic nonsense mutations in the hMSH6 gene but retain activity of the hMLH1 and hMSH2 proteins (Table 1). The majority of the other cell lines lack either hMLH1 (HCT116, DU145, SW48, and AN3CA) or hMSH2 (SK-UT-1B and LoVo), with the exception of LS411N, which was not yet studied for mutations in these genes, and LS180, which was found to be wild type for these genes as well as for hPMS1 and hPMS2 (59). hMSH6 is required for the repair of single-nucleotide mismatches but is less critical for the repair of DNA strand slippage. hMLH1 and hMSH2 are required for the repair of both types of mismatches. Thus, hMSH6-mutant cells are only partially repair-deficient (60). We showed previously that the mutator phenotype of hMSH6-mutant cells is distinct from that of hMLH1 or hMSH2-mutant cells (61). It is characterized by a significantly lower frequency of deletion/insertion frameshift mutations in mononucleotide (61) and dinucleotide (19) repeats. Thus, because many DNA DSB repair genes contain mononucleotide repeats, the probability of their mutational inactivation in the cells with hMSH2 or hMLH1 defects is higher than in cells that are only deficient in hMSH6. This suggests that the DLD-1 and HCT-8 cell lines may carry only a single aberration in the DNA DSB repair machinery, the heterozygous MRE11 gene mutation, which is not sufficient to abrogate the repair activity in these cells to the extent of the increased sensitivity to bleomycin.

There may be an alternative explanation to the differences in bleomycin sensitivity between MSI-positive and -negative tumors. It was reported previously that the presence of the mutated p53 gene is associated with the cell resistance to the toxic effect of DNA DSB producing agents (62). MSI-positive and -negative tumors differ in the frequency of p53 gene mutations with the latter carrying the mutated p53 more frequently (4). However, the presence of p53 mutations did not correlate with bleomycin sensitivity in our experiments. DU145 MSI-positive cells carry p53 gene mutations (Table 1) but are hypersensitive to bleomycin (Fig. 2). The DLD-1 and HCT-8 cell lines, which have a similar genetic background but differ in p53 status, exhibit a similar sensitivity to bleomycin, characteristic of the MSI-negative cells.

Besides the cell lines that carry a homozygously mutated DNA DSB repair gene, two cell lines, SK-UT-1B and DU145, which carry concomitant heterozygous mutations in three different DNA DSB repair genes, ATR, RAD50, MRE11, or DNA-PKcs, RAD50, MRE11, respectively, were also hypersensitive to bleomycin. We hypothesize that concomitant heterozygous mutations in DNA DSB repair genes can cause an accumulative haploinsufficiency in the cellular repair activity (13), which would be reflected as an increased sensitivity to bleomycin. We show that mutational inactivation of a single allele of the DNA-PKcs gene in the subclones of the HCT-8 cell line make the cells sensitive to bleomycin at lower doses of the DNA-PK inhibitor LY294002 compared with the original HCT-8 cells (Fig. 5). This strongly indicates that inactivation of one of the two alleles of the DNA-PKcs gene decreases the overall DNA DSB repair activity in the cells.

Haploinsufficiency of enzymatic pathways can be a frequent phenomenon in MMR-deficient cells (13, 63). Heterozygous mutations in different genes participating in the same pathway can accumulate...
faster than homologous mutations in a single gene of the pathway. Even if the first heterozygous mutation does not produce haploinsufficiency, the subsequent heterozygous mutations that occur in the other gene-members of the same pathway may lead to an increase in haploinsufficiency and a corresponding decrease in the functional efficiency of the pathway. Because of the high mutation rate in MMR-compromised cells, such cascade accumulation of mutations may frequently occur during tumor development (or during the division of a tumor precursor cell) in pathways that are not even important for tumorigenesis and the functional deficiency of which does not give the cells any growth advantage. Such impairment of biochemical functions in MSI-positive cells can be used for specific therapies of MMR-deficient cancer. Treatment with bleomycin or other DNA DSB producing agents, including γ-radiation, can be one of such specific therapies.

A cascade accumulation of mutations in DNA repair pathways can lead to a deepening of the microsatellite mutator phenotype through, as we showed previously, further inactivation of the DNA MMR (39) and to a broadening of the mutator phenotype through cumulative haploinsufficiency of the DNA DSB repair, which can cause chromosomal instability. A cascade accumulation of heterozygous mutations in tumorigenic pathways during the division of MMR-deficient cells can lead to a cumulative haploinsufficiency of the cellular tumor suppressor function without even the complete inactivation of any particular tumor suppressor gene. This may be one of the most fundamental differences between the mechanisms of tumorigenesis of MMR-deficient and proficient cancers.

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