DNA mismatch repair and p53 function are major determinants of the rate of development of cisplatin resistance

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Abstract

As opposed to factors that control sensitivity to the acute cytotoxic effect of cisplatin, little is known about the factors that determine the rate at which resistance develops. This study examined how loss of p53 or DNA mismatch repair (MMR) function affected the rate of development of resistance to cisplatin in human colon carcinoma cells during sequential cycles of cisplatin exposure that mimic the way the drug is used in the clinic. We used a panel of sublines molecularly engineered to express either the MMR- and p53-proficient phenotype or singly or doubly deficient phenotypes. Loss of either MMR or p53 alone increased the rate of development of resistance to cisplatin by 1.8- and 2.4-fold, respectively; however, loss of both MMR and p53 increased the rate by 4.8-fold. Inhibition of DNA polymerase ζ by suppression of the expression of its REV3 subunit eliminated the increased rate of development of resistance observed in the MMR-deficient cells. Loss of p53 or MMR increased the steady-state level of *REV3* and of *REV1* mRNA; loss of both functions increased these levels much further by a factor of 20.2-fold for *REV3* and 10.3-fold for *REV1*. The basal level of homologous recombination measured using a reporter vector was 1.3- to 1.7-fold higher in cells that had lost either p53 or MMR function, and 2.6-fold higher in cells that had lost both. In the p53- and MMR-proficient cells, cisplatin induced a 17-fold increase in homologous recombination even when the recombining sequences that did not contain cisplatin adducts; the magnitude of induction was even greater in cells that had lost either one or both functions. We conclude that separate from effects on sensitivity to the acute cytotoxic effect of cisplatin, loss of MMR, especially when combined with loss of p53, results in rapid evolution of cisplatin resistance during sequential rounds of drug exposure that is likely mediated by enhanced mutagenic translesion synthesis. The DNA damage response activated by cisplatin is accompanied by a p53- and MMR-dependent increase in homologous recombination even between adduct-free sequences. [Mol Cancer Ther 2006;5(5):1239–47]

Introduction

The clinical efficacy of cisplatin is often limited by the rapid development of resistance, but the factors that determine the risk of developing resistance are largely unknown. Cisplatin forms adducts in DNA and is a mutagen in mammalian cells (1–4). Current evidence suggests that cisplatin resistance arises through a combination of mutagenesis that generates highly cisplatin-resistant clones in the surviving population and enrichment of these variants by subsequent cycles of cisplatin treatment. We have been interested in whether functions that protect against genomic instability modulate the risk of acquiring resistance to cisplatin. DNA mismatch repair (MMR) and p53 are of particular interest because their function is lost frequently in human tumors and, in some tumor types, loss of MMR directly causes cisplatin resistance possibly by altering adduct detection (5, 6). MMR is also of interest because cisplatin treatment enriches for cells that have lost MMR both *in vitro* (7) and *in vivo* (8). Even a single exposure to cisplatin is sufficient to enrich for MMR-deficient cells preexisting in a tumor cell population despite the fact that the magnitude of the resistance produced by loss of MMR is only in the range of 2-fold (7). Loss of either MMR or p53 function sensitizes cells to the ability of cisplatin to generate highly drug-resistant variants in the surviving population (4).

A recent study examined how MMR and p53 interact to maintain genomic integrity in the presence of the mutagenic stress induced by cisplatin (9). Sensitivity to the cytotoxic and mutagenic effect of cisplatin was assessed using a panel of sublines of the MMR-deficient HCT116 colon carcinoma cells in which MMR function had been restored by transfer of a copy of MLH1 on chromosome 3, or in which p53 function had been disabled by expression of human papillomavirus-16 E6. Loss of MMR or p53 alone had only a small effect on sensitivity to the mutagenic effect of cisplatin as measured by the appearance of drug-resistant variants (up to 2.4-fold), whereas loss of both p53 and MMR had a more profound effect (up to 6.5-fold). Loss of both p53 and MMR increased the basal frequency of insertion/deletion mutations detected by a shuttle vector–based assay to a greater extent than loss of either alone.

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In association with cisplatin-induced injury, loss of p53 or MMR alone resulted in 1.2- and 1.7-fold more mutations, whereas loss of both resulted in a 5.1-fold increase in mutant frequency. Examination of the effect of loss of p53 and/or MMR on the cisplatin-induced cell cycle checkpoint activation, p53 induction, ability of the cell to tolerate adducts in its DNA, and the rate of disappearance of Pt from genomic DNA indicated effects of the loss of p53 and/or MMR on all of these variables. These results indicated that p53 and MMR cooperate to limit the mutagenic potential of cisplatin in the colon cancer cells.

In *Escherichia coli*, DNA damage produces a well-defined SOS response in which large numbers of mutations are produced by specialized DNA polymerases that are capable of inserting nucleotides opposite lesions in DNA but do so with a high error rate (reviewed in refs. 10, 11). However, mutations also occur in sequences that contain no damage (12). A homologous set of specialized DNA polymerases exists in human cells and includes the B-family member polymerase ζ and the Y-family members polymerase η, polymerase ι, polymerase κ, and REV1 (13). These enzymes are characterized by their low fidelity when copying undamaged templates, their lack of 3′ to 5′ proofreading activity, and their ability to bypass lesions that block progression of the replicative DNA polymerases (13). Although cisplatin triggers sister chromatid exchange (2), most of the mutations generated by cisplatin seem to result from bypass replication across the adducts that it produces in DNA by polymerase ζ and members of the Y-family of mutagenic DNA polymerases (14, 15). However, it is not known whether cisplatin can induce an error-prone state equivalent to the SOS response in *E. coli*, during which mutations are generated in genes that do not contain any adducts.

In this study, we directly measured the effect of the loss of p53 and MMR function on the rate at which cisplatin resistance developed during sequential cycles of cisplatin exposure. We report here that cells that have lost these functions develop resistance to cisplatin at an increased rate. The enhanced rate observed in MMR-deficient cells is dependent on polymerase ζ and is associated with increased steady-state expression of REV3, the catalytic subunit of polymerase ζ, and of REV1 as well as a marked increase in the ability of cisplatin to induce homologous recombination between DNA sequences that do not contain adducts. The results provide strong evidence that p53 and MMR control the rate of resistance development and provide additional insight into the mechanism of this effect.

Materials and Methods

Cell Lines

A total of seven sublines of the human colorectal adenocarcinoma cell line HCT116 were used in this study, each engineered to express a different phenotype. The variants were HCT116+ch3, HCT116, HCT116+ch3/E6, HCT116/E6, HCT116+ch3-kdREV3, and HCT116-kdREV3 representing $MMR^+/p53^+$, $MMR^-/p53^+$, $MMR^+/p53^-$, $MMR^-/p53^-$, $MMR^+/p53^+/REV3^-$, and $MMR^-/p53^+/REV3^-$ phenotypes, respectively. In addition, a 7th variant in which both alleles of p53 had been deleted (designated $HCT116/p53^{-/-}$) was used to confirm results obtained when E6 was used to disable p53 function. The hMLH1-deficient HCT116 was obtained from the American Type Culture Collection (Manassas, VA); HCT116 contains a hemizygous mutation in hMLH1 resulting in a truncated, nonfunctional protein (16) but is proficient in p53 function (17, 18). A subline complemented with chromosome 3 (HCT116+ch3) was obtained from Drs. C.R. Boland and M. Koi (University of Michigan Medical School, Ann Arbor, MI). The chromosome 3–complemented cells were competent in DNA MMR (19). HCT116 and HCT116+ch3 sublines expressing papillomavirus E6 (identified here as HCT116/E6 and HCT116+ch3/E6) were obtained from Drs. D.A. Boothman and M. Meyers (University of Wisconsin-Madison, Madison, WI) (20). In these cells lines, there is constitutive high-level expression of the *human papillomavirus type-16 E6* gene, which stimulates the degradation of p53 through a ubiquitin pathway, disrupted p53 function (20). The HCT116+ch3 and HCT116 cell lines were engineered as previously reported (21) to suppress *hREV3* mRNA by stable expression of a short hairpin interfering RNA targeted to hREV3, the catalytic subunit of DNA polymerase ζ, to generate REV3 knockdown HCT116+ch3-kdREV3 and HCT116-kdREV3 sublines. HCT116 cells in which both p53 alleles were deleted by targeted homologous recombination (17), designated $HCT116/p53^{-/-}$, were obtained from Dr. Bert Vogelstein (The Johns Hopkins Medical Institutions, Baltimore, MD). These were maintained in McCoy's 5A media (Irvine Scientific, Santa Ana, CA) supplemented with 10% FCS and penicillin/streptomycin. All other cell lines were maintained in Iscove's modified Dulbecco medium (Irvine Scientific) supplemented with 2 mmol/L L-glutamine and 10% heat-inactivated fetal bovine serum. The chromosome-complemented lines were maintained in medium containing 400 μg/mL geneticin (Life Technologies, Inc., Grand Island, NY). The cell lines expressing papillomavirus E6 were cultured in medium supplemented with 80 μg/mL hygromycin B (Boehringer Mannheim, Indianapolis, IN), and the REV3 knockdown cell lines were grown in the medium supplemented with 1 μg/mL puromycin.

Reagents

Cisplatin was gifts from Bristol-Myers Squibb (Princeton, NJ). A stock solution of 3.33 mmol/L cisplatin in 0.9% NaCl was stored in the dark at room temperature.

Relative Rate of Development Resistance to Cisplatin

The rate at which a cell population became resistant to cisplatin during repeated cycles of 1-hour exposures to cisplatin was determined by measuring the IC_{50} for cisplatin using a clonogenic assay following each round of selection. The cisplatin concentration used for selection was the IC_{90} for the population under study. For each round of selection, 10^6 cells were exposed to cisplatin for 1 hour. When the cells had recovered to 90% confluence, an aliquot was used to determine cell number and the slope of the cisplatin concentration-survival curve in a

clonogenic assay, and another aliquot was again exposed to cisplatin. Total cell number and plating efficiency was determined at each step; this information, along with the exact number cells subcultured, was used to calculate population doubling according to the following equation: population doubling = (ln[total number of cells] − ln[number of cells plated × plating efficiency]) / ln2. The rate of acquisition of resistance to cisplatin was then calculated by plotting the slope of the cisplatin concentration-survival curve as a function of population doubling. The slope of the latter plot yields the rate of relative resistance development.

Quantification of *hREV1* and *hREV3* mRNA by Real-time PCR

Total RNA was extracted with TRIzol^R reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and random primers. Real-time PCR was done using the BIO-RAD iCycler iQ detection system in the presence of SYBR-Green I dye (Bio-Rad laboratory, Inc., Hercules, CA). The forward and reverse primers for *REV1* were 5′-AAGGCT-GATGCAATCG-3′ and 5′-CCACCTGGACATTGTCAA-GAATAA-3′, respectively. The forward and reverse primers for *REV3* were 5′-TGATGTCTTCAGCTGGTAT-CATGA-3′ and 5′-CCGCCCTTCAGGTTCACTT-3′, respectively. These were used for amplification with an iCycler protocol consisting of a denaturation program (95°C for 3 minutes), amplification and quantification program repeated 40 times (95°C for 10 seconds and 55°C for 45 seconds), and melting curve analysis. A melt-curve analysis immediately followed amplification and was executed using 95°C for 1 minute then 55°C for 1 minute followed by 80 repeats of heating for 10 seconds, starting at 55°C with 0.5°C increments. The data were analyzed by using the comparative C_t method, where C_t is the cycle number at which fluorescence first exceeds the threshold. The ΔC_t values from each cell line were obtained by subtracting the values for 18S C_t from the sample C_t. A 1 unit difference of C_t value represents a 2-fold difference in the level of mRNA.

Measurement of the Frequency of Extrachromosomal Homologous Recombination

Homologous recombination was assayed by determining the extent of recombination between two green fluorescent protein (GFP) sequences in plasmid DNA as previously described (22). The pBHRF vector contains an intact ''blue'' variant of GFP (EBFP) that includes a ≈300 nucleotide sequence with perfect homology to a second truncated nonfunctional copy of GFP. In the absence of homologous recombination within the vector, only EBFP is expressed; however, homologous recombination between the EBFP and truncated GFP sequences creates a functional GFP, and if this occurs, the cell expresses GFP and EBFP, which is expressed from other plasmids in the cell that have not undergone recombination. Cells were seeded into six-well plates overnight and then exposed to 20 μmol/L cisplatin for 1 hour. The surviving cells were then transfected with pBHRF at various time points thereafter using siPORT *XP-1* transfection agent (Ambion, Inc., Austin, TX) in the presence of serum according to the manufacturer's instructions. Four hours after transfection, BoosterExpress reagent (Gene Therapy Systems, Inc., San Diego, CA) was added, and the cells were then analyzed by two-color flow cytometry 48 hours after the start of the transfection. The recombination frequency was calculated as RF = [(EBFP$^+$ and GFP$^+$) + (GFP$^+$)] / [(EBFP$^+$ and GFP$^+$) + (GFP$^+$) + (EBFP$^+$)], where (EBFP) and (GFP) represent the number of blue and green fluorescent cells, respectively, in the sample.

Results

Effect of the Loss of MMR and/or p53 on the Rate of Development of Cisplatin Resistance

The emergence of drug resistance in a population during repeated cycles of cisplatin exposure may be due to enrichment for preexisting resistant clones, cisplatin-induced generation of new resistant variants, or some combination of both. We have previously shown that loss of MMR and/or p53 function increases the ability of cisplatin to generate drug-resistant variants in the surviving population, including variants that are very highly resistant to cisplatin itself (9). If this is central to the emergence of acquired cisplatin resistance in the whole population, then inactivation of MMR and/or p53 would be expected to increase the rate at which resistance emerges. We measured the rate of development of resistance in the whole population of four closely related cell lines (HCT116+ch3, HCT116, HCT116+ch3/E6, HCT116+ch3/E6, and HCT116/E6) molecularly engineered to exhibit an MMR$^+$/p53$^+$, MMR$^-$/p53$^+$, MMR$^+$/p53$^-$, or MMR$^-$/p53$^-$ phenotype, respectively, all of which grow at similar rates. The HCT116+ch3 cells are MMR proficient due to the wild-type *MLH1* gene on the inserted chromosome 3 and have normal p53 function. In the HCT116+ch3/E6 and HCT116/E6 cells, the function of p53 was disabled due to the expression of human papillomavirus-16 E6. For simplicity of presentation, these cell lines will subsequently be identified by their phenotype in this article. Starting with 500,000 cells, each population was exposed to an IC_{90} concentration of cisplatin for 1 hour, and the exposure was repeated as soon as log phase growth resumed. After each round of drug treatment, the sensitivity of the whole population to cisplatin was measured by determining the IC_{50} in a clonogenic assay that measured survival over 2 logs of cell kill as a function of cisplatin concentration and comparing this to the IC_{50} of the same subline before the start of cisplatin selection. Figure 1 shows that cisplatin resistance emerged relatively slowly in the MMR$^+$/p53$^+$ cells. Based on the ratio of the slopes of the plot of resistance as a function of population doubling, loss of either MMR or p53 function alone moderately increased the rate of development of resistance to cisplatin by 1.8- and 2.4-fold ($P < 0.05$) above that observed in the MMR$^+$/p53$^+$ cells. However, loss of both MMR and p53 increased the rate of development of resistance by 4.8-fold ($P < 0.01$).

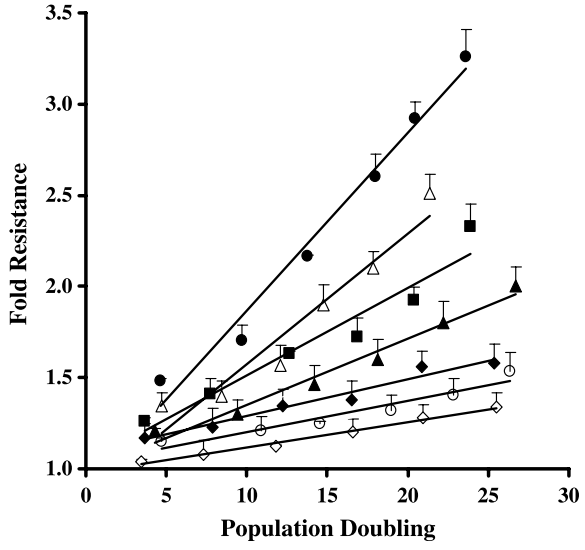

Figure 1. Effect of loss of MMR, p53, and polymerase ζ function on the rate of development of cisplatin resistance. ♦, $MMR^+/p53^+$ (HCT116 + ch3) cells; ▲, $MMR^-/p53^+$ (HCT116) cells; ■, $MMR^+/p53^-$ (HCT116 + ch3/E6) cells; ●, $MMR^-/p53^-$ (HCT116/E6) cells; △, HCT116/$p53^{-/-}$ cells;◇, $MMR^+/p53^+/REV3^-$ (HCT116 + ch3-kdREV3); ○, $MMR^-/p53^+/REV3^-$ (HCT116-kdREV3). *Points,* mean of three independent measurements; *bars,* SE.

These results show that both p53 and MMR control the rate of development of resistance and suggest that when their function is lost cisplatin induces even greater degrees of genomic instability with the result that more drug-resistant variants are generated that can become enriched in the population by subsequent rounds of cisplatin exposure.

To confirm the results obtained when expression of E6 was used to disable p53 function, the rate of development of cisplatin resistance was also measured in the HCT116/$p53^{-/-}$ subline in which both *p53* alleles had been somatically deleted. As shown in Fig. 1, cisplatin resistance was acquired 3.5-fold more rapidly in the HCT116/$p53^{-/-}$ cells than in the $MMR^+/p53^+$ HCT116+ch3 cells ($P < 0.01$) and 2.0-fold more rapidly than in the parental HCT116/$p53^{+/+}$ cells. Interestingly, degradation of p53 by E6 expression had larger effect on the rate of development of cisplatin resistance than *p53* allele deletion. This observation is consistent with the concept that E6 can affect the function of other cellular proteins, some of which may also modulate the acquisition of cisplatin resistance.

Effect of Disabling Polymerase ζ on the Rate of Development of Cisplatin Resistance in MMR-Proficient and MMR-Deficient Cells

Deficiency of MMR in colon carcinoma cell lines has been reported to result in increased replicative bypass across cisplatin adducts (23). Our recent studies showed that loss of MMR enhanced the polymerase ζ–dependent generation of drug-resistant variants in the surviving population (21). To determine whether this translated into an effect on the rate of development of resistance at the population level, the rate of development of cisplatin resistance in HCT116+ch3 and HCT116 cell lines was compared with their isogenic counterparts HCT116+ch3-kdREV3 and HCT116-kdREV3, in which polymerase ζ function had been disabled by knockdown of the expression of REV3. In the $MMR^+/p53^+$ cells, knockdown of hREV3 had only a minor effect on the rate of development of cisplatin resistance; it reduced the rate by 1.2 ± 0.15-fold (±SE; $P > 0.05$). In contrast, as shown in Fig. 1, knockdown of hREV3 in the $MMR^-/p53^+$ cells had a more profound effect on the rate of development of cisplatin resistance and decreased the rate by 2.6 ± 0.06-fold ($P < 0.01$). This result indicates that the increased rate of development of resistance seen in the MMR-deficient cells is dependent on polymerase ζ function. This suggests that loss of MMR is associated with increased translesional synthesis.

Effect of the Loss of MMR and p53 on the Steady-State Level of *REV1* and *REV3* mRNA

In yeast, mutagenic translesion synthesis mediated by polymerase ζ and REV1 accounts for essentially all DNA damage–induced mutagenesis (24). REV1 enhances the activity of polymerase ζ (25) and assists in loading it onto stalled replication forks (26). We have shown that suppression of the expression of either REV3, the catalytic subunit of polymerase ζ, or of REV1, reduces the mutagenicity of cisplatin and slows the rate of development of resistance by factors of 3.0- and 2.8-fold, respectively (14, 15). This suggests that the mutagenicity of cisplatin in human cells is also largely due to mutagenic translesional synthesis across cisplatin adducts. If this is the case, then the question arises as to whether the enhanced mutagenicity of cisplatin in cells that have lost MMR or p53 function is due to enhanced translesion synthesis. At the present time, no antibody is available with sufficient avidity and specificity to permit accurate Western blot analysis of hREV3 protein levels. Thus, real-time PCR was used to measure the steady-state levels of both *REV3* and *REV1* mRNA level. As shown in Fig. 2A, loss of MMR function alone increased the steady-state endogenous *REV3* mRNA level by 2.5-fold ($P < 0.01$), whereas loss of p53 alone increased *REV3* mRNA level by a factor of 3.4-fold ($P < 0.01$). However, the steady-state level of *hREV3* mRNA in the doubly deficient $MMR^-/p53^-$ cells was 20.2-fold higher ($P < 0.01$) than in the $MMR^+/p53^+$ cells. As shown in Fig. 2B, a similar pattern was observed for *REV1* mRNA expression where loss of MMR alone, p53 alone, or both increased the steady-state *REV1* mRNA level by 3.7-, 6.6-, and 10.3-fold, respectively, compared with that in the $MMR^+/p53^+$ cells. In the case of REV3, no information is available as to whether the mRNA level predicts the protein level, but in the case of REV1, there is a close association (15). Thus, these results are consistent with the concept that proficiency of MMR and p53 function directly influences the abundance of both REV3 and REV1 and thus the mutagenic translesion synthesis capacity.

Induction of Homologous Recombination in Nonadducted DNA Sequences

Cisplatin adducts in DNA are known to trigger recombination. However, homologous recombination occurring

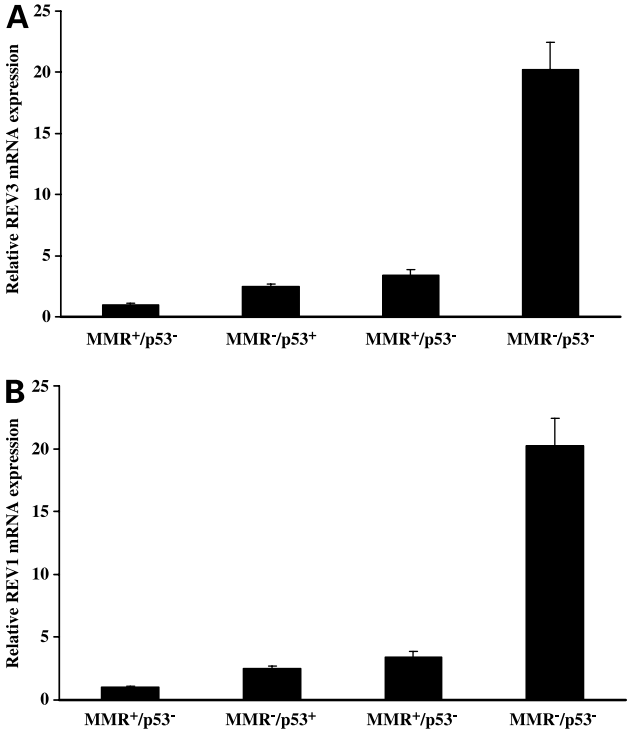

Figure 2. Relative REV3 (**A**) and REV1 (**B**) mRNA levels in $MMR^+/p53^+$ (HCT116 + ch3), $MMR^-/p53^+$ (HCT116), $MMR^+/p53^-$ (HCT116 + ch3/E6), and $MMR^-/p53^-$ (HCT116/E6) cells. The expression of REV3 and REV1 mRNA was measured by quantitative real-time PCR using the BIO-RAD iCycler iQ detection system in the presence of SYBR-Green I dye. The relative expression level of REV3 and REV1 was determined by normalizing the ΔC_t value to the HCT116 + ch3 cell value. The level of REV3 and REV1 mRNA in the HCT116 + ch3 cells was arbitrarily set to 1.

between sequences that do not contain adducts must result from activation of a generalized response. As a surrogate measure of whether cisplatin induces a SOS-like response in human cells, we sought to determine whether cisplatin induces homologous recombination between DNA sequences in which adducts have not formed. This was accomplished by introducing the recombining sequences into the cell after free cisplatin had disappeared. To map the time course of this effect, we transfected the recombination-sensitive reporter vector pBHRF into either untreated $MMR^+/p53^+$ cells or the same cells at various times starting 6 hours after a 1-hour exposure to an IC_{90} concentration of cisplatin (20 μmol/L) and then measured the frequency of recombination that occurred over the ensuing 48 hours. The ability of this vector to detect differences in recombination frequency, such as those that accompany loss of BRCA1 or p53 function, has already been documented (22). Our prior studies showed that by 6 hours after the end of drug exposure, no free intracellular cisplatin remains; thus, when introduced ≥6 hours after the end of the drug exposure, the pBHRF should contain no adducts. As shown in Fig. 3, the recombination frequency rose quickly during the initial phases of the injury response and peaked at 17.1 ± 1.9-fold above baseline in cells transfected at 24 hours. It then gradually decreased until, in cells transfected at 96 hours, it was at the same level as in cells transfected at 6 hours. Thus, in cells that are both MMR and p53 proficient, cisplatin induced a large increase in homologous recombination between extrachromosomal sequences that contained no adducts. The time course of this effect corresponded very closely to the time course of perturbation of cell cycle phase progression induced by cisplatin in these same cells, wherein peak G_2-M arrest occurred at 24 hours and had largely resolved by 96 hours (9).

Effect of the Loss of MMR and/or p53 on the Frequency of pBHRF Recombination Induced by Cisplatin

To determine how the loss of either MMR or p53 function modulated basal and cisplatin-induced homologous recombination, the pBHRF vector was transfected into the panel of $MMR^+/p53^+$, $MMR^+/p53^-$, $MMR^+/p53^+$, or $MMR^-/p53^-$ cell lines. Based on the time course of the response in the $MMR^+/p53^+$ cells, a transfection time point of 24 hours after the end of a 1-hour exposure to 20 μmol/L cisplatin was selected for comparison of the magnitude of induction in each cell type. As shown in Fig. 4, the basal recombination frequency in non–cisplatin-treated cells was lowest for the $MMR^+/p53^+$ cells. Loss of MMR did not increase the basal frequency significantly, but loss of p53 increased it by 1.7-fold ($P < 0.05$), and loss of both MMR and p53 increased it by 2.6-fold ($P < 0.01$). When the $MMR^+/p53^+$ cells were injured with cisplatin, the recombination frequency increased by a factor of 17-fold over baseline. However, loss of MMR alone, p53 alone, or both increased recombination by factors of 27-, 26-, and 20-fold,

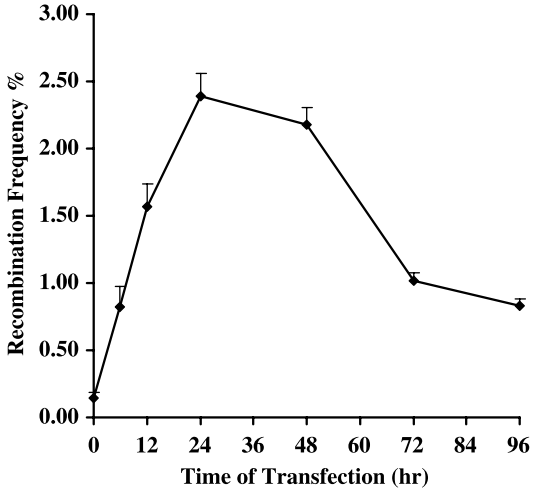

Figure 3. Frequency of pBHRF recombination as a function of time after cisplatin exposure in $MMR^+/p53^+$ (HCT116 + ch3) cells. The cells were exposed to 20 μmol/L cisplatin for 1 h and thoroughly washed remaining free drug in the medium. Any free intracellular cisplatin was allowed to efflux for 6 h, and the reporter vector was then transfected into the cells at either 6, 12, 24, 48, 72, 96 h after the end of the cisplatin exposure. *Points,* mean of three experiments done with duplicate culture; *bars,* SE.

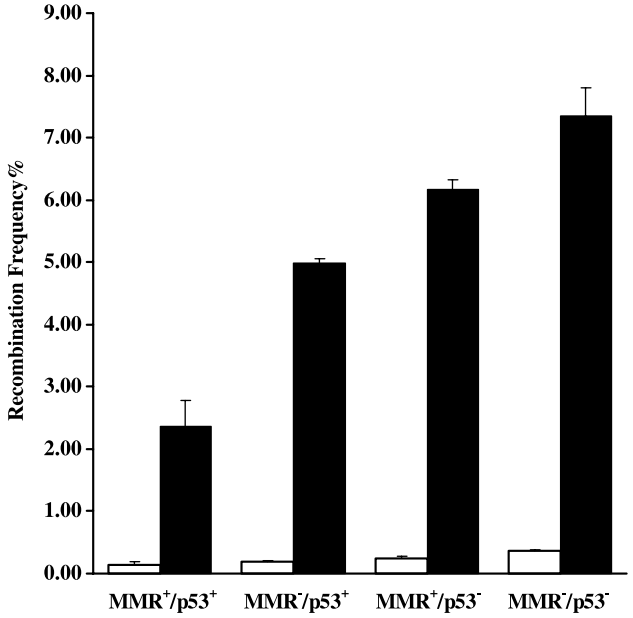

Figure 4. Effect of loss of MMR and p53 on cisplatin-induced frequency of homologous recombination in pBHRF sequences not containing cisplatin adducts. *Columns*, mean of three experiments each done with duplicate cultures; *bars*, SE.

respectively. The cisplatin-induced increase in recombination frequency was statistically significant in all four cell types. Thus, the magnitude of the induction of recombination among DNA sequences not containing cisplatin adducts was influenced significantly by loss of either MMR or p53, and the induced recombination frequency was highest when both functions were disabled.

To confirm the effect of the loss of p53 function, additional studies were carried out in the truly isogenic HCT116/p53$^{+/+}$ and HCT116/p53$^{-/-}$ pair of cell lines. The recombination frequencies measured when pBHRF was transfected into the HCT116/p53$^{+/+}$ and HCT116/p53$^{-/-}$ cell 24 hours after a 1-hour exposure to 20 μmol/L cisplatin are shown in Fig. 5. Although the basal recombination frequency was 2.3-fold ($P < 0.05$) higher in HCT116/p53$^{-/-}$ cells, cisplatin exposure generated an additional 1.5-fold increase ($P < 0.05$) in recombination frequency in the HCT116/p53$^{-/-}$ cells in which neither copy of the *p53* gene was functional. Thus, disabling p53 by expressing E6 had a somewhat more permissive effect on pHBRF recombination, that deletion of both p53 alleles suggests that E6 may affect additional pathways that modulate mutagenicity. These results show that a functional *p53* tumor suppressor gene operates to maintain genomic stability, as measured by pBHRF recombination, during the DNA damage response triggered by cisplatin.

Discussion

Among human tumors, there is substantial heterogeneity in both initial clinical response and the rate of development of resistance to cisplatin during treatment. It is likely that the factors that control sensitivity to the acute cytotoxic effect of cisplatin are quite different from those that control the risk of developing resistance when the tumor is subjected to sequential cycles of drug exposure. Although MMR and p53 function have small and inconsistent effects on sensitivity to the cytotoxic effect of cisplatin in various tumor models (27–38), the studies reported here indicate that they are both important determinants of the rate at which resistance evolves with repeated cycles of drug exposure. The results provide further evidence that the mechanism by which MMR and p53 regulate the rate of development of drug resistance is through affects on mutagenic translesion synthesis. In addition to burdening the genome with adducts that can serve as hotspots for the generation of mutations, we found that cisplatin activates a mechanism that markedly increases the frequency of homologous recombination among DNA sequences that do not contain adducts. The enhanced recombination is also significantly modulated by MMR and p53 function.

The single most important finding reported here is that loss of MMR, especially when combined with loss of 53 function, results in the more rapid evolution of cisplatin resistance during sequential rounds of drug exposure that mimic the way the drug is used in the clinic. Although both MMR and loss of p53 are well documented to cause cancer syndromes and various types of genomic instability, the current study provides direct evidence that they actually control the rate of development of cisplatin resistance at the population level. The increase in the rate of development of resistance parallels very closely the enhanced mutagenicity of cisplatin in cells that have lost either MMR or p53 and the marked increase in mutagenicity when both are lost (9).

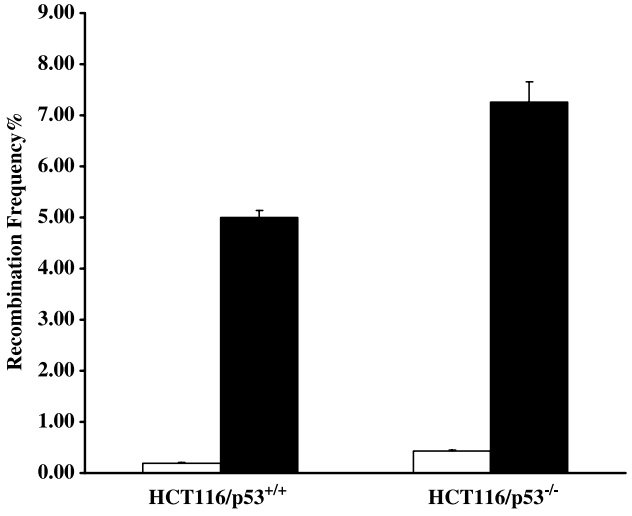

Figure 5. Frequency of pBHRF homologous recombination in the HCT116/p53$^{+/+}$ and HCT116/p53$^{-/-}$ cells 24 h after exposure to 20 μmol/L cisplatin for 1 h. Harvest procedures and treatment conditions were identical to those in Figs. 3 and 4.

Thus, there is a close association between the ability of cisplatin to generate variants in the surviving population that are highly resistant to cisplatin itself and the rate at which resistance evolves at the population level ($r = 0.98$ for correlation between rate of development resistance to cisplatin and frequency of cisplatin-induced mutation to 6-thioguanine resistance; ref. 9).

What is the mechanism by which loss of MMR or p53 enhances the mutagenicity of cisplatin? There is now a substantial body of evidence pointing to mutagenic translesion synthesis across cisplatin adducts in DNA as the major source of cisplatin-induced mutations (14, 15, 21). Five specialized DNA polymerases have been implicated in mutagenic translesion synthesis in mammalian cells, including polymerase ζ, polymerase η, polymerase ι, polymerase κ, and REV1; others may contribute as well (39). Among these, we have previously shown that reduction in the expression of either REV3, the catalytic subunit of polymerase ζ, or of REV1 reduces the ability of cisplatin to generate highly drug-resistant variants in the surviving population and reduces the rate at which cisplatin resistance evolves at the population level (14, 15). In addition, exposure to cisplatin directly up-regulates the expression of REV3 and REV1, thus potentially facilitating mutagenic translesion synthesis across adducts not immediately removed by nucleotide excision repair (14, 15). Such overexpression of polymerase ι and polymerase κ has already been shown to increase mutation rates even in uninjured mammalian cells (40–42).

There is now also evidence that p53 and MMR directly influence the mutagenic translesion synthesis pathway. First, we have previously documented that loss of p53 or MMR, but particularly loss of both, increases the tolerance of the cell for adducts in its genome, a feature characteristic of cells with increased translesion synthetic capacity (43). Second, in the current study, we found that steady-state levels of both REV1 and REV3 were higher in cells that had lost either p53 or MMR or both. Third, when REV3 expression is suppressed, the enhanced mutagenicity of cisplatin in the MMR-deficient cells is eliminated (21). Finally, suppression of REV3 eliminated the enhanced rate of development of resistance observed when MMR was lost. Thus, enhanced translesion synthesis provides a reasonable explanation for the increased mutagenicity of cisplatin in MMR- or p53-deficient cells. However, p53 also modulates transcription-coupled nucleotide excision repair, which is a determinant of sensitivity to cisplatin (44) and may also affect other DNA repair pathways that operate on double-strand breaks or interstrand cross-links that may contribute to enhanced mutagenesis. It is noteworthy that for each of the four variables measured in the current study (the rate of development of cisplatin resistance, the induction of homologous recombination, the level of REV 1, and the level of REV3), loss of MMR or p53 each independently had an effect, and this was similar to the effect that MMR and p53 have on sensitivity to the mutagenic effect of cisplatin (9). In all cases, the effect is substantially greater when both functions are lost. Thus, both MMR and p53 govern the mechanism that determines the rate of development of resistance, and their functions are not epistatic in that loss of both produces larger effects than loss of either alone.

Mutagenic translesion synthesis across cisplatin adducts by preexisting specialized DNA polymerases provides an explanation for the source of mutations, and recombinational activity focused at adducted sites provides a mechanistic explanation for the ability of cisplatin to enhance sister chromatid exchange (2). However, the results reported here indicate that exposure to cisplatin triggers activation of repair activities even at sites in DNA that do not contain adducts. As noted above, we have reported that REV1 and REV3 are up-regulated by cisplatin exposure (14, 15), and in a study of the cellular response to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, REV3 was previously implicated in the generation of mutations in non-adducted as well as adducted DNA templates (45). This may result from the action of the translesion synthesis pathway on abnormal or distorted replication forks not associated with drug-induced adducts but due to endogenous oxidative DNA damage (46). Distinguishing between events resulting from repair activities operating on an adduct in DNA and a generalized increase in error-prone or error-free repair that affects the entire genome is a challenge. We have previously reported that loss of p53 or MMR function increased the basal frequency of mutation in a vector that reports on microsatellite instability, and that exposure to cisplatin increased the mutation frequency in a p53- and MMR-dependent manner, but this system did not allow rigorous exclusion of the possibility that cisplatin adducts were introduced into the vector itself (9). We approached this challenge in the current study by monitoring homologous recombination between sequences introduced into the cell after free cisplatin had disappeared. Cisplatin markedly up-regulated this activity, and the time course of the up-regulated activity matched closely the development and subsequent resolution of cisplatin-induced perturbation of cell cycle progression (9), peaking when the reporter vector was introduced at 24 hours after the drug exposure and partially resolving by 96 hours. The fact that the recombination frequency increased during the period where, if there had been any free cisplatin left in the cell it would have been expected to be decreasing, provides evidence that the recombination observed was not being driven by the formation of adducts in the pBHRF vector. Cisplatin reacts with a variety of targets in the cell in addition to DNA; thus, it is not certain that the enhanced homologous recombination is triggered solely by DNA damage. However, whatever the mechanism of up-regulation, it was substantially affected by both MMR and p53 function. As was the case for all the other variables discussed above, loss of either MMR or p53 alone individually increased the recombinational response, but loss of both more than doubled the rate.

Whether there is a direct link between the permissive effect that loss of p53 or MMR have on cisplatin-induced homologous recombination and their effect on the rate of

development of cisplatin resistance is not yet clear. Homologous recombination is important to the survival of cells following cisplatin exposure (47–49), and it may be essential for repair of interstrand cross-links (50–52). However, homologous recombination between nonadducted DNA sequences is believed to be a largely error-free repair mechanism. Thus, although it may protect against activation of apoptosis, it seems unlikely that enhanced homologous recombination contributes to cisplatin-induced mutagenesis. However, there are now several lines of evidence indicating a link between translesion synthesis and homologous recombination or other mechanisms of interstrand cross-link repair. Polymerase ζ has recently been shown to participate in a homologous recombination-independent interstrand cross-link repair in yeast (53) and mammalian cells (54). Overexpression of polymerase β, a polymerase that functions largely in base excision repair but can bypass cisplatin adducts (55), increases homologous recombination in a Rad51-dependent manner (56). Using the same pBHRF reporter system, we found that knocking down the expression of either REV1 or REV3 substantially reduced steady-state and cisplatin-induced pBHRF recombination (14, 15). This suggests a role for hREV1, polymerase ζ (REV3 and REV7 subunits), or one of the other proteins with which hREV1 interacts in homologous recombination, consistent with another recent report (57). Thus, the increased pBHRF recombination frequency observed in cisplatin-treated cells may be a secondary result of the up-regulation of proteins whose primary function is to mediate translesion synthesis. The finding that cisplatin exposure enhances pBHRF recombination suggests that cisplatin up-regulates this putatively nonmutagenic repair mechanism, an effect expected to improve the ability of the cell to survive the DNA damage produced by this agent.

It is important to point out several limitations of the experimental model that mandate caution when interpreting the results reported here. First, despite the fact that the HCT116+ch3 and HCT116 cells have been very useful for investigation of the mechanism of MMR, they are not isogenic. In addition to contributing a wild-type copy of the *MLH1* gene, the extra copy of chromosome 3 inserted in the HCT116+ch3 cells may contribute other genes whose activity is important to the effects of cisplatin. Second, expression of the E6 protein affects other functions in addition to p53 that could be important to the cisplatin injury response.

It is worth emphasizing again that the single most important finding reported here is that loss of MMR or p53 alone, but particularly loss of both functions, very substantially increased the rate at which cisplatin resistance developed during sequential cycles of drug exposure. There is a high probability that most tumors treated with cisplatin contain at least a few cells in which both functions have been lost, and that these can sow the seeds of therapeutic failure. The available data provide the outline of a credible explanation for the mechanism that underlies this effect, but further confirmation that this mechanism is translesion synthesis is needed. Clinical studies of the role of MMR and p53 have largely focused on whether they control the initial responsiveness of tumors. Irrespective of their effects on sensitivity to the acute cytotoxic effect of cisplatin, the results reported here suggest that MMR and p53 regulate the risk of development of resistance. Because the function of these two pathways is often lost in human cancers, they are prime candidates as determinants of therapeutic outcome.

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