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Decreased Cisplatin Damage-Dependent DNA Synthesis in Cellular Extracts of Mismatch Repair Deficient Cells

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ABSTRACT. The proficiency of both nucleotide excision repair (NER) and DNA mismatch repair (MMR) influences cellular sensitivity to cisplatin (*cis*-diamminedichloroplatinum). To gain further insight into how MMR may influence platinum drug sensitivity, the effect of loss of MMR on repair synthesis was measured *in vitro* by a commonly used method that relies on whole-cell extracts to drive [α-^{32}P]dATP incorporation into cisplatin-damaged plasmid DNA. Extracts evaluated include those from cells with or without functional hMLH1 (HCT116+ch2 versus HCT116+ch3, respectively) and hMSH2 (HEC59 versus HEC59+ch2, respectively). Loss of MMR in the HCT116 system was associated with a 2.8-fold reduction in cisplatin damage-specific DNA synthesis, whereas it was associated with a 3.0-fold reduction in the HEC59 system, suggesting that a decrease in the ability to repair cisplatin-damaged DNA accompanies loss of MMR. An *in vitro* DNA excision assay that utilized a substrate containing a site-specific cisplatin adduct was performed. Using this highly NER-specific assay, no significant difference was apparent between the extracts derived from NER-proficient versus -deficient cells. These and other data lead us to suggest that the increase in apparent repair synthesis in platinum-damaged plasmids by extracts from MMR-proficient versus -deficient cellular extracts may reflect a distinct and possibly adverse DNA synthetic process rather than productive NER. BIOCHEM PHARMACOL **57**;8:861–867, 1999.

KEY WORDS. DNA mismatch repair; cisplatin; drug resistance

The main function of the MMR§ system is to scan newly synthesized DNA and remove mismatches that result from nucleotide incorporation errors made by the DNA polymerases. This DNA repair mechanism has received considerable attention due to the fact that mutations in the MMR genes underlie the syndrome of hereditary nonpolyposis colorectal cancer, which is characterized by very high rates of early-onset colorectal and other cancers. The majority of these families carry a germline mutation in one allele of an MMR gene, and the second allele is disabled by somatic mutation in tissues prone to malignant transformation. To date, six genes have been identified whose products participate in MMR: *hMLH1*, *hMSH2*, *hMSH3*, *hMSH6*, *hPMS1*, and *hPMS2*. Loss of MMR also occurs in a wide variety of sporadic cancers [1].

In addition to causing genomic instability, loss of MMR causes drug resistance. It has been reported that loss of MMR is associated with low-level resistance to cisplatin [2], and that the selection of cells in culture for resistance to this drug often yields cell lines that have lost a functional MMR system [2, 3]. This is of substantial concern because of the facts that cisplatin is so widely used for the treatment of human malignancies, and that the magnitude of the resistance that accompanies loss of MMR is sufficient to account for the failure of treatment in model systems [4]. Recently, models have been developed that allow the impact of MMR on drug sensitivity to be studied in some detail. Among these are cells that have been molecularly engineered to create sublines that are either MMR-proficient or -deficient. The human colon carcinoma cell line HCT116 is MMR-deficient due to a deletion in one allele of *hMLH1* and a mutation in the other copy of the gene [5]. The HCT116+ch3 subline was rendered MMR-proficient by transfer of a complete chromosome 3, which contains a functional copy of the *hMLH1* gene. The HCT116+ch2 cell line was created by transferring chromosome 2 into the parental HCT116 cell line; this cell line serves as a control for the microcell-mediated chromosome transfer process, since chromosome 2 does not contain the *hMLH1* gene [6]. The HCT116+ch2 cells are 2.1-fold resistant to cisplatin and 1.3-fold resistant to carboplatin relative to the HCT116+ch3 cells [7]. Hence, the MMR-deficient subline demonstrates low-level cisplatin resistance compared with

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§ *Abbreviations*: MMR, DNA mismatch repair; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; DTT, dithiothreitol; and NER, nucleotide excision repair.

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the MMR-proficient subline. A similar system has allowed the impact of hMSH2 function on platinum drug sensitivity to be determined. HEC59 is a human endometrial carcinoma cell line that is MMR-deficient due to mutations in both alleles of *hMSH2* [5]. In HEC59+ch2 cells, the MMR deficiency has been corrected by transfer of a full-length chromosome 2, which contains the *hMSH2* gene [8]. The MMR-deficient HEC59 cells are 1.8-fold resistant to cisplatin and 1.5-fold resistant to carboplatin relative to the MMR-proficient subline HEC59+ch2 cells. In contrast, neither of the pairs of MMR-proficient and -deficient cells differed with respect to their sensitivity to oxaliplatin [7].

These data are consistent with the possibility that the MMR system serves as a detector of cisplatin and carboplatin adducts in DNA but not of oxaliplatin adducts. MSH2 alone, and in combination with hMSH6, has been shown to bind to cisplatin 1,2-d(GpG) intrastrand adducts with high efficiency [9–11]. Additionally, hMSH2- and hMLH1-containing protein–DNA complexes were observed by using mobility shift assays when nuclear extracts of the MMR-proficient cell lines were incubated with DNA platinated with cisplatin but not with oxaliplatin [7], confirming a difference in the capacity of MMR-deficient and -proficient cell lines to detect the lesions produced by cisplatin versus oxaliplatin. These data suggested that MMR recognition of damage may trigger an apoptotic death pathway, rendering cells with intact MMR more sensitive to DNA damage.

Interestingly, MMR deficiency has been associated recently with a defect in transcription-coupled repair [12, 13]. Transcription-coupled repair refers to the enhanced nucleotide excision repair observed to occur in the transcribed strand of active genes. Cyclobutane pyrimidine dimers caused by UV light are removed by transcription-coupled repair, and this type of NER may be important in the repair of other types of DNA damage [14]. The association of MMR deficiency with both cisplatin resistance and a decrease in the activity of an NER pathway seems counterintuitive since there is extensive evidence to support a relationship between perturbations in NER genes, the major pathway by which platinum–DNA adducts are removed, and increased sensitivity to platinum drugs. This issue prompted us to investigate the relationship between MMR capacity and cisplatin DNA adduct repair. We compared the ability of the MMR-proficient and -deficient HCT116 and HEC59 sublines to carry out repair synthesis on a cisplatin-damaged plasmid substrate, using a commonly used *in vitro* repair synthesis assay that is generally considered to measure NER. Results obtained with these experiments led us to examine these extracts using a more specific measurement for the cisplatin–DNA adduct NER, an *in vitro* DNA excision assay.

MATERIALS AND METHODS

Chemicals and Reagents

Cisplatin was obtained from Bristol Myers Squibb. Cell culture reagents were obtained from Life Technologies, Inc., except for the fetal bovine serum, which was obtained from Atlanta Biologicals. All other chemicals were obtained from the Sigma Chemical Co. unless otherwise indicated.

Cell Lines

Cell lines were maintained at 37° in 5% CO_2 in Iscove's modified Dulbecco's medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 μg/mL of streptomycin, 100 U/mL of penicillin, and 2 mM glutamine. The chromosome-complemented sublines were maintained in 0.4 mg/mL (HCT116+ch2 and HCT116+ch3) or 0.6 mg/mL (HEC59+ch2) of geneticin.

Preparation of Whole-Cell Extracts

Extracts were prepared essentially by the method of Manley *et al.* [15]. Cells were plated in four 245 × 245 × 20 mm culture dishes and harvested at 48–72 hr (80–90% confluency). After rinsing the plates with cold PBS, the cells were scraped into 5 mL of hypotonic lysis buffer containing protease inhibitors [10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 μg/mL each of leupeptin, pepstatin, and chymostatin]. After 20 min on ice, the cells were lysed by 30 strokes with a Dounce homogenizer. An equal volume of 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 2 mM DTT, 25% sucrose, and 50% glycerol, and 1.25 mL of neutralized, saturated ammonium sulfate were then added with gentle mixing, and stirring was continued for 50 min on ice. The mixture was then centrifuged at 125,000 *g* for 3 hr at 2°. Proteins were precipitated from the resultant supernatant by adding 0.33 g/mL of ammonium sulfate and were neutralized with 10 μL of 1 N NaOH per g of ammonium sulfate added. The precipitate was collected by centrifugation and dialyzed for 16 hr against 1 L of 25 mM HEPES-KOH (pH 7.9), 0.1 mM KCl, 12 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, and 17% glycerol. Insoluble material was removed by centrifugation, and the extract was frozen in small aliquots and stored at −80°.

In Vitro *DNA Repair Synthesis Assay*

Repair reactions were performed essentially by the method of Wood *et al.* [16]. Closed circular plasmids were prepared by growth in *recA*, *endA* AG-1 *Escherichia coli* (Stratagene) followed by alkaline lysis, purification on Qiagen columns according to the manufacturer's guidelines, and two consecutive rounds of sucrose gradient centrifugation [5–20% sucrose, 25 mM Tris–HCl (pH 7.5)], 1.0 M NaCl, 5 mM EDTA]. Centrifugations were carried out at 86,000 *g* at 2° for 18 hr. Before the second centrifugation, the pBS plasmid was treated at a concentration of 0.1 mg/mL with 16 μM cisplatin for 4 hr at 37°. Under these conditions, the resulting lesion density was approximately 25 Pt lesions/plasmid as determined by atomic absorption spectrometry.

Fifty-microliter repair reactions contained 300 ng pBS plasmid damaged with cisplatin and 300 ng undamaged pCRII plasmid, 45 mM HEPES-KOH (pH 7.8), 70 mM KCl, 7.4 mM $MgCl_2$, 0.5 mM DTT, 0.4 mM EDTA, 2 mM ATP, 20 μM each of dGTP, dCTP, and TTP, 8 μM dATP, 2 μCi [α-^{32}P]dATP, 23 mM phosphocreatine, 2.5 μg creatine phosphokinase Type I (Sigma), 3.4% glycerol, 18 μg BSA, and 150 μg extract protein. Reactions were incubated for 3 hr at 30° and stopped by adding EDTA to 20 mM. Plasmid DNA was purified from the reaction mixture and linearized with *Eco*RI. Samples were electrophoresed in a 1% agarose gel containing 0.5 μg/mL of ethidium bromide. The gel was photographed with Polaroid type 55 positive/negative film, dried, and autoradiographed. Phosphoimaging was done using the Fuji Macbas 2000 imaging system from Fuji Medical Systems, and radioactivity was determined in some cases by cutting the bands from the gel and subjecting the solubilized gel pieces to liquid scintillation counting. Recovery was determined by scanning the photographic negative using the AMBIS optical imaging system.

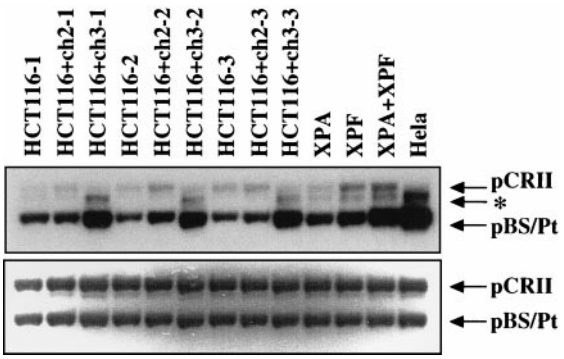

FIG. 1. ***In vitro*** **repair synthesis assay of extracts prepared from the HCT116 cell lines. Three independent whole-cell extracts of cell lines HCT116, HCT116+ch2, and HCT116+ch3 were prepared, and 150 μg of each was used in an** ***in vitro*** **DNA repair assay. XPA, XPF, XPA+XPF (75 μg of each), and HeLa extracts were included as controls. The top panel is the autoradiogram and the bottom panel is a photograph of the ethidium bromide stained gel. The presence of a third band that migrated between the pCRII and pBS/Pt bands was noted in the top panel of Fig. 1 and is labeled "*". This may correspond with a faint band migrating underneath pCRII in the photograph depicted in the lower panel.**

Single Lesion Excision Assay

The *in vitro* excision experiments were done essentially by methods described by Moggs *et al.* [17]. Briefly, the 24-mer 5′-TCTTCTTCTGTGCACTCTTCTTCT-3′ was reacted with cisplatin and gel purified to isolate the major product of this reaction, the oligonucleotide containing a 1,3-intrastrand d(GpTpG)-cisplatin cross-link. This oligonucleotide or a purified undamaged oligonucleotide was used to prime the plus strand of a modified M13mp18 molecule (M13mp18GTGx), and the second strand was synthesized by incubation in a reaction mixture containing 10 mM Tris–HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 600 μM each of dATP, dCTP, dGTP, and TTP, T4 DNA polymerase gp43 subunit (Promega), and T4 DNA ligase (NEB). Closed circular DNA then was isolated by CsCl/EtBr density gradient centrifugation. Fifty-microliter reaction mixtures contained 250 ng of the modified M13mp18 molecule containing a site-specific 1,3-intrastrand d(GpTpG)-cisplatin cross-link (Pt-GTG) or an undamaged control substrate (Con-GTG) and 125 μg of whole-cell extract protein. For complementation experiments, 5 μL of a 20 ng/mL preparation of purified ERCC1-XPF protein was added per reaction. The substrate and protein were incubated in a buffer containing 45 mM HEPES-KOH (pH 7.8), 70 mM KCl, 7.5 mM $MgCl_2$, 0.9 mM DTT, 0.4 mM EDTA, 2 mM ATP, 20 μM each of dATP, dCTP, dGTP, and TTP, 40 mM phosphocreatine, 2.5 μg creatine phosphokinase, 3.4% glycerol, and 18 μg BSA for 30 min at 30°. Purified DNA was electrophoresed through a 12% denaturing polyacrylamide gel, and the DNA was transferred onto a nylon membrane (Hybond N^+, Amersham) by capillary transfer. Fixed membranes were incubated in 40 mL of buffer containing 7% SDS, 10% polyethylene glycol 8000, 250 mM NaCl, 130 mM potassium phosphate buffer (pH 7.0), and 100 pmol of ^{32}P-labeled oligonucleotide with the sequence 5′-GAAGAGT GCACAGAAGAAGAGGCCTGG-3′. After washing in 1× SSC (0.15 M sodium chloride, 0.015 M sodium citrate) with 0.1% SDS, the membranes were exposed to x-ray film (X-OMAT AR, Kodak). Quantitation was done by densitometry using the AMBIS optical imaging system (AMBIS).

RESULTS

Whole-cell extracts were prepared in triplicate from each of the MMR-proficient and -deficient cell lines. These extracts were examined for *in vitro* DNA repair activity as measured by the [α-^{32}P]dATP incorporated into the cisplatin-damaged pBS plasmid relative to that incorporated into undamaged pCRII plasmid. Little extract-to-extract variation in activity was observed (3–20%). Both the MMR-deficient HCT116 and HCT116+ch2 human colon carcinoma cells yielded extracts with relatively low levels of cisplatin damage-dependent DNA synthesis in this assay. Cellular extracts from the MMR-proficient HCT116+ch3 cells had 2.8-fold greater activity [395.0 ± 12.9 (SD) fmol dAMP incorporated] than the MMR-deficient HCT116+ch2 [141.6 ± 23.5 (SD) fmol dAMP incorporated, $P = 8.1 \times 10^{-5}$] or HCT116 cells [118.5 ± 12.9 (SD) fmol dAMP incorporated, $P = 1.24 \times 10^{-5}$] (Fig. 1). Similarly, cellular extracts prepared from the MMR-proficient HEC59+ch2 cells had 3.0-fold more activity [381.0 ± 77.9 (SD) fmol dAMP incorporated] than the MMR-deficient HEC59 cells [128.2 ± 10.1 (SD) fmol dAMP incorporated, $P = 5.1 \times 10^{-3}$] (Fig. 2). Extracts from the MMR-deficient cell lines had activity in the range of extracts of cell lines from patients in two complementation groups of the nucleotide excision repair-deficient

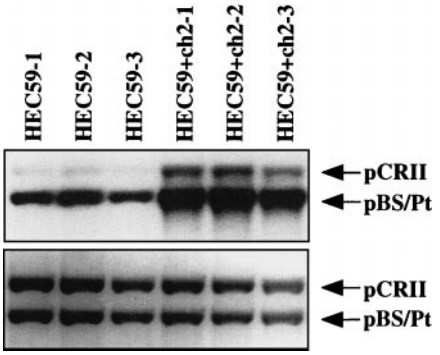

FIG. 2. ***In vitro*** **repair synthesis assay of extracts prepared from the HEC59 cell lines. Three independent whole-cell extracts of cell lines HEC59 and HEC59+ch2 were prepared and 150 μg of each was tested in an** ***in vitro*** **DNA repair assay. The top panel is the autoradiogram, and the bottom panel is a photograph of the ethidium bromide stained gel.**

syndrome xeroderma pigmentosum (XPA and XPF) (150.4 and 238.7 fmol dAMP incorporated, respectively). Mixing of the deficient XPA and XPF extracts while keeping the total protein constant accomplished complementation of repair synthesis (524.8 fmol dAMP incorporated). An extract from an HeLa cell line served as a positive control in this assay (735.9 fmol dAMP incorporated) (Fig. 1). The data obtained from the *in vitro* DNA repair synthesis assays are summarized in Fig. 3. The presence of a third band, which migrated between the pCRII and pBS/Pt bands, was noted in the top panel of Fig. 1 and is labeled "*". This may correspond with a faint band migrating underneath pCRII in the photograph depicted in the lower panel. This band's relative intensity suggests that it is related to the pBS/Pt plasmid, and its migration corresponds to a nicked open-circle form of the plasmid. It is possible that damage-specific synthesis in these plasmids has disrupted the *Eco*RI site.

Figure 4 shows the results of a single lesion excision assay that utilizes a closed circular DNA molecule containing a site-specific 1,3-intrastrand d(GpTpG) cisplatin adduct incubated with whole-cell extracts from the HCT116 and HEC59 sets of MMR-proficient and -deficient cell lines. Reaction products were separated on a 12% denaturing polyacrylamide gel, which was then transferred onto a nylon membrane and probed with a ^{32}P-labeled oligonucleotide complementary to the region of the substrate that is removed during the dual incision process of NER. The autoradiograph shown in Fig. 4 revealed no correlation between mismatch repair status and ability to complete the NER dual incision event. Dual incision of the Pt-(GTG) substrate results in products that range in size from 25 to 30 nucleotides with the most predominant being 26 nucleotides in length [17]. The approximate size of these bands was verified by comparison with the migration of an end-labeled, platinated 24-mer used in the construction of the single lesion substrate (data not shown). Control samples are shown in lanes 6–9 (shown at a longer exposure time). Extracts prepared from XPA and XPF cells showed no indication of dual incision activity, as expected. Dual incision activity was reconstituted by mixing these extracts, while keeping the total protein constant. Lane 9 is an HCT116 extract incubated with Con-GTG, a substrate devoid of the Pt-GTG lesion. Table 1 summarizes the data obtained with two independently prepared extracts of each cell line.

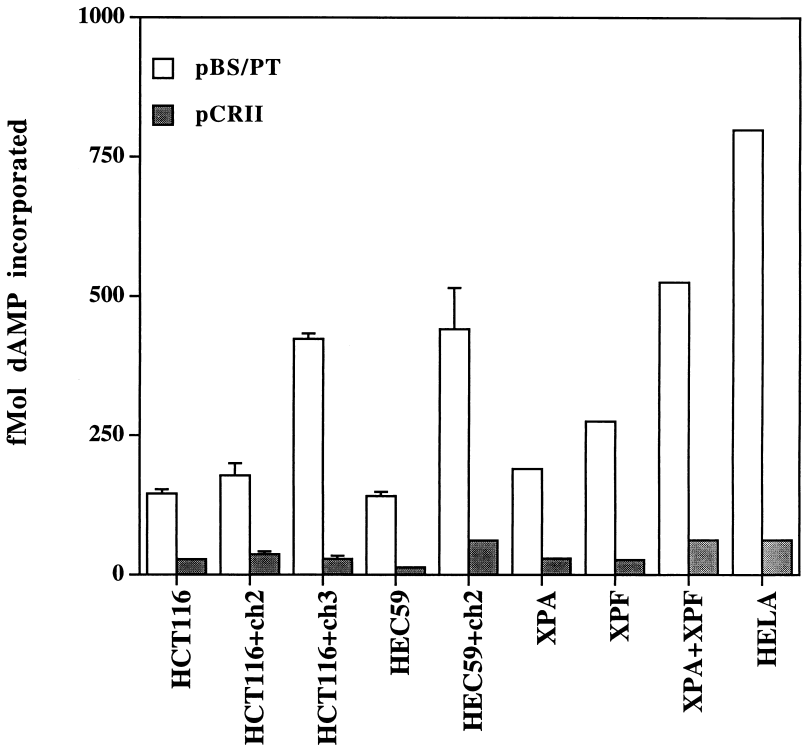

FIG. 3. Quantitation of ***in vitro*** **DNA repair data. Triplicate extract counts per minute were averaged, corrected for recovery and decay of the isotope, and converted to femtomoles dAMP incorporated into the plasmid DNA. Vertical bars are SD, N = 3.**

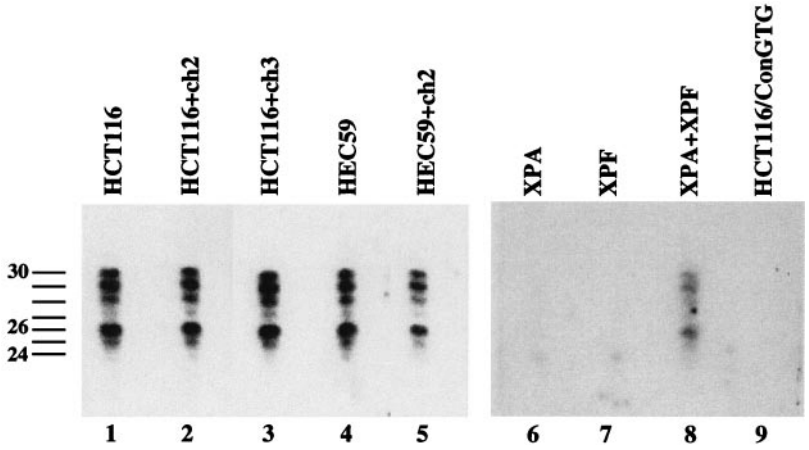

FIG. 4. ***In vitro*** **DNA excision assay of extracts prepared from the HCT116 and HEC59 sets of cell lines. Shown is the autoradiograph of the Southern blot in which oligonucleotides formed during the dual incision reaction were transferred onto a nylon membrane and probed with a ^{32}P-labeled complementary probe. The sizes of the excised oligonucleotides are indicated and were determined by comparison with the mobility of the platinated 24-mer used in the synthesis of the substrate. Representative reactions using 125 μg of extract protein prepared from cell lines HCT116, HCT116+ch2, HCT116+ch3, HEC59, and HEC59+ch2 are shown (lanes 1–5, respectively). Results from reactions using extracts XPA (125 μg), XPF (125 μg), and XPA+XPF (62.5 μg of each) are shown at a longer exposure time (lanes 6–8, respectively). Results from reactions in which 125 μg of an HCT 116 extract was incubated with a substrate devoid of the Pt-GTG adduct are shown, also at a longer exposure time (lane 9).**

DISCUSSION

The results of the initial studies using the *in vitro* DNA repair synthesis assay suggested that loss of MMR due to dysfunction of either *hMLH1* or *hMSH2* is associated with reduced DNA repair synthesis as measured *in vitro* by the ability of whole-cell extracts to drive [α-^{32}P]dATP incorporation into cisplatin-damaged plasmids. The assay used was designed to measure NER [16], and NER is the dominant mechanism by which intrastrand cisplatin adducts are removed from DNA [18]. The implication is that MMR proficiency is required for full NER-mediated removal of cisplatin adducts. This is consistent with the observation that loss of MMR can impair transcription-coupled repair in some systems [12, 13]. However, NER activity can be reconstituted in cell-free systems using purified proteins that do not include the MMR proteins [19, 20], and several other studies have concluded that NER and MMR function independently [21, 22]. Furthermore, loss or impairment of NER due to several different types of molecular lesions results in hypersensitivity to cisplatin. Thus, one would predict that if loss of MMR significantly impaired the NER-mediated removal of cisplatin adducts, the MMR-deficient cell should be hypersensitive to this drug. However, loss of MMR has been shown to produce low-level resistance to cisplatin in multiple different cell systems [2–4, 23]. Most importantly, here we have demonstrated that there is no correlation between MMR status and NER dual incision activity. This assay utilizes a site-specifically damaged, single lesion substrate, which allows for detection of the oligonucleotides formed during the dual incision reaction to be detected by a Southern blot methodology, and, hence, is highly specific for NER [22]. In addition, the presence of MMR had no impact on platinum DNA adduct removal as measured by atomic absorption spectroscopy.*

TABLE 1. Quantitation of single lesion excision assay of MMR-deficient cell lines and their chromosome-complemented counterparts

Extract	Average relative intensity
HCT116	85.2 ± 3.5
HCT116+ch2	114.3 ± 26.6
HCT116+ch3	51.5 ± 19.9
HEC59	50.9 ± 28.3
HEC59+ch2	49.9 ± 18.4

Average relative intensity was derived from densitometric scanning of the autoradiograph using the AMBIS system. Values are means ± range, N = 2.

A possible explanation for the observation of increased cisplatin damage-specific DNA synthesis in the extracts prepared from MMR-proficient cell lines that does not correlate with increased dual incision activity is that the MMR-driven [α-^{32}P]dATP incorporation is due to a DNA synthetic process that is distinct from NER and that is detrimental to survival. Although the *in vitro* DNA repair synthesis assay used here is generally considered a valid way to assess NER, any process that results in new DNA synthesis in the plasmid substrate is measurable by the assay. This assay is believed to measure NER activity in terms of the extent of [α-^{32}P]dATP incorporation into the gap created by excision of a DNA lesion, e.g. a cisplatin DNA adduct. However, it is possible that, in addition to NER, MMR is also contributing to the creation of gaps in cisplatin adducted DNA. In the case of NER, it is clear that the adducted strand is cut at approximately the ninth phosphodiester bond 3′ and at the sixteenth phosphodiester bond 5′ to the adduct, and the gap resulting from the

* Cenni B, Kim HK, Bubley GJ, Fink D, Aebi S, Teicher BA, Howell SB and Christen RD, Manuscript submitted for publication.

removal of the intervening fragment is then filled in by DNA polymerases and ligated to complete a repair process that removes the adduct from the DNA [17]. The MMR system may also be creating gaps in either the adducted or non-adducted strand, and the assay may be measuring incorporation of [α-^{32}P]dATP into these gaps as well as those created by NER-mediated removal of cisplatin-DNA adducts. This MMR-mediated DNA synthetic process, however, may not result in the correction of the damage. This concept is also supported by data on MMR-mediated processing of MNNG or 6-thioguanine adducts. Current evidence suggests that following recognition of either MNNG or 6-thioguanine in DNA, the MMR system recognizes a distortion in the undamaged DNA strand and attempts to repair that distortion by creating a nick in that strand, which then is converted to a gap by an exonuclease activity. When the gap is filled, because the adduct persists in the template strand, either replication ceases at the site of the adduct, or, if bypass replication occurs, a mismatch is created that is once again recognized by the MMR system, leading to another round of futile attempts at repair. Such futile cycles of attempted repair have been hypothesized to be the basis for MNNG and 6-thioguanine toxicity, and loss of MMR is a mechanism of resistance to these drugs [24]. Furthermore, replicative bypass of cisplatin–DNA adducts has also been demonstrated [25]. It has been reported very recently that defects in both *hMLH1* and *hMSH6* result in increased cisplatin resistance and decreased replicative bypass as measured by steady-state elongation assays [26]. Hence, the *in vitro* DNA repair synthesis assay data we present may reflect incorporation of [α-^{32}P]dATP into gaps created by both NER and MMR, in which case loss of MMR would reduce [α-^{32}P]dATP incorporation and apparent repair synthesis activity.

The results reported here are consistent with the concept that the MMR system serves as a detector for cisplatin-induced DNA damage [7, 9–11], and extends this model to suggest that the MMR system, having recognized the presence of an adduct, creates nicks in the DNA. Hence, a functional MMR system not only may have a role in initiating apoptosis but also may create DNA damage and, therefore, directly aid cisplatin-mediated cell death.

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