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Advances in Brief

Dependence of the Cytotoxicity of DNA-Damaging Agents on the Mismatch Repair Status of Human Cells

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Abstract

Mismatch repair (MMR) deficiency was reported to increase resistance of mammalian cells to killing by several genotoxic substances. However, although MMR-deficient cells are ~100-fold more resistant to killing by S_N1 type methylating agents than MMR-proficient controls, the sensitivity differences reported for the other agents were typically <2-fold. To test whether these differences were linked to factors other than MMR status, we studied the cytotoxicities of mitomycin C, chloroethylcyclohexyl nitrosourea, melphalan, psoralen-UVA, etoposide, camptothecin, ionizing radiation, and *cis*-dichlorodiaminoplatinum (cisplatin) in a strictly isogenic system. We now report that MMR deficiency reproducibly desensitized cells solely to cisplatin.

Introduction

The mismatch repair (MMR) system plays an important role in the maintenance of genomic stability by correcting replication errors that escape processing by the proofreading activity of DNA polymerases and by controlling the fidelity of homologous recombination. Cells in which the MMR system has been inactivated display microsatellite instability, a phenotype identified in tumors of several different origins, both hereditary and sporadic (1). MMR status has also been reported to influence the response of cells to a number of genotoxic agents and chemotherapeutics. However, whereas the link between MMR defects and resistance to *N*-methyl-*N*-nitrosourea, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, temozolomide, 6-thioguanine, and *cis*-dichlorodiaminoplatinum (cisplatin) is generally accepted (2), the effect of MMR status on the response of mammalian cells to other chemotherapeutic agents has been questioned. Thus, MMR-deficient cells were found to be more resistant to ionizing radiation (IR) in some laboratories (3, 4) but not in others (5, 6). Loss of MMR has furthermore been reported to be associated with an increased resistance to topoisomerase (topo) poisons, especially the topo II inhibitors doxorubicin, epirubicin, and mitoxanthrone (7), yet others described a sensitization to the same class of substances in the absence of MMR (8–10). MMR-deficient clones derived from the human cancer cell lines HeLa and Raji were reported to be more sensitive to chloroethylcyclohexyl nitrosourea (CCNU) and mitomycin C (MMC; Refs. 11, 12), and this phenotypic trait was observed also in some MMR-deficient tumor cell lines (11). However, as in the case of IR, some cancer cell lines (11) and one MMR-deficient clone derived from an ovarian carcinoma cell line (13) displayed an increased resistance to CCNU. Because MMR-deficient cells have a mutator phenotype, their genomes are in a state of continuous flux during growth in tissue culture. Thus, clones derived from the same source could acquire slightly different phenotypes in different laboratories, and it is therefore formally possible that the discrepancies described above might be linked with these unknown phenotypic differences rather than with the MMR status of the cells. Given that many of the agents listed above are routinely used in cancer chemotherapy, the role of the MMR system in their efficacy must be elucidated, otherwise, their deployment in the treatment of tumors with microsatellite instability might have deleterious consequences. To address this issue while avoiding the above pitfalls, we studied the cytotoxicity of several DNA cross-linking agents, topo inhibitors, and IR in a strictly isogenic system in which the MMR status is regulated by doxycycline (Dox) and where the switching from MMR-proficient to -deficient status and *vice versa* does not involve clonal selection.

Materials and Methods

Cells. The 293T Lα cell line was established in our laboratory (14). It was derived from the hMLH1-deficient human embryonic kidney 293T cells by stable transfection with a vector carrying the hMLH1 cDNA under the control of the inducible Tet-Off expression system. The cells were grown in DMEM with Eagle salts (Life Technologies, Inc., Gaithersburg, MD), supplemented with 10% Tet System approved fetal bovine serum (Clontech, Palo Alto, CA), 2 mM L-glutamine (Life Technologies, Inc.), 100 IU/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc.), 100 μg/ml Zeocin (Invitrogen, San Diego, CA), and 300 μg/ml Hygromycin B (Roche Molecular Biochemicals, Basel, Switzerland). To obtain cells completely free of the MMR protein hMLH1 (293T Lα^-), the cells were transferred for at least 7 days to a medium containing 50 ng/ml Dox (Clontech). Fresh Dox was added every second day. To induce hMLH1 expression (293T Lα^+), the cells were transferred to a medium without Dox, the medium was changed the following day, and the cells were cultivated for at least 6 more days. Expression of hMLH1 in these cells fully restored MMR proficiency. All incubations were at 37°C in humidified 5% CO_2 atmosphere. The cells were free of *Mycoplasma* contamination.

Genotoxic Treatments. The cells were plated 1 day before treatment, and the exponentially growing attached cells were then treated either for 1 h with MMC (Sigma), melphalan (Sigma), CCNU (Lomustine; Bristol-Myers Squibb), or cisplatin (Fluka) or for 3 h with etoposide (Sigma) or camptothecin (Sigma) at the indicated concentrations at 37°C. After drug exposure, the cells were washed with PBS and incubated in fresh medium. Because of the short half-life (~1 h) of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (Sigma) in aqueous solution, the medium was not changed after the treatment. In the case of psoralen treatment, 5000 cells were plated in 60-mm plates 1 day before treatment. The cells were then washed with PBS, incubated for 10 min in the dark at 37°C with 1 μM 4,5′, 8-trimethyl-psoralen (Aldrich), in PBS/1 mM $MgCl_2$, and then irradiated with 366 nm UV light (UV-A). They were then washed, fresh medium was added, and the cells were incubated for 5 days at 37°C. The medium was then removed, the cells were washed twice with PBS and stained with 1 ml of staining solution [0.1% crystal violet/1 mM $MgCl_2$/10 mM potassium phosphate (pH 7.4)/250 mM sucrose] for 30 min. Cells were then rinsed and lysed with 10% acetic acid, and absorbance was measured at 595 nm. Cell viability was expressed relative to that of cells incubated with 4,5′, 8-trimethyl-psoralen without UV irradiation.

MMR Assays. *In vitro* MMR reaction was carried out as described previously (14). Briefly, a circular 3188-bp DNA molecule containing a one nucleotide loop within the recognition site of *Bgl*II restriction endonuclease and a nick 5′ from the mispair was incubated with nuclear protein extract. The

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substrate and unrepaired DNA molecule is resistant to *Bgl*II digestion, and successful MMR reaction reconstitutes the *Bgl*II site, making the DNA cleavable.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide (MTT) Assays. A total of 2000 cells was plated in 96-well plates, treated with different concentrations of genotoxic agents the next day, and incubated for 4 or 5 more days. MTT (Sigma) solution was then added at a final concentration 0.9 mg/ml, and the plates were incubated for 5 h at 37°C. One volume of lysis solution was then added [20% SDS, 50% dimethylformamide (pH $<$ 4.7)], and the plates were incubated overnight at 37°C. The solubilized formazan was quantified spectrophotometrically at 570 nm, using the V_{max} microplate reader (Molecular Devices, Sunnyvale, CA). The absorbance values were plotted against drug concentrations, and IC_{50} values were calculated from the regression curve.

Clonogenic Assays. Cells at ~50% confluency were either treated with CCNU, melphalan or cisplatin, or were irradiated (Philips PW2184/00-Monitor SN4) with the indicated doses. They were then harvested and plated at various dilutions. At the same time, 300 untreated cells were plated to assess plating efficiency. Visible colonies were counted after 10 days.

Cell Cycle Analysis. Cells, both attached and floating, were harvested, counted, washed with PBS, fixed with 70% ethanol, and stored up to 1 week at 4°C. The cells were then washed with PBS, incubated in PBS containing RNase A (100 μg/ml; Sigma) for 1 h at 37°C, stained with 20 μg/ml propidium iodide (Sigma), and incubated on ice in the dark for 30 min. DNA content was analyzed by Coulter EpicsFlow Cytometer (Beckman Coulter, Inc., Fullerton, CA).

Results and Discussion

The 293T Lα cell line was derived from the hMLH1-deficient human embryonic kidney 293T cells by stable transfection with a vector carrying the hMLH1 cDNA under the control of the inducible Tet-Off expression system. In the absence of Dox, these cells express hMLH1 and are MMR proficient (293T Lα^+). When Dox is added to the medium, hMLH1 expression is shut off, and the cells become MMR deficient (293T Lα^-; Fig. 1*A*; Ref. 14). In this strictly isogenic setting, any differences in sensitivity of the 293T Lα^+ and 293T Lα^- cells to chemotherapeutic agents can be ascribed to the different MMR status of the cells because GeneChip analysis detected no significant differences in the transcriptomes of the 293T Lα^- and -Lα^+ cells, other than the change in *hMLH1* mRNA levels (15).

In our earlier study (14), we could show that the MMR-proficient 293T Lα^+ cells were 125-fold more sensitive to killing by the S_N1 type methylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (Table 1) and arrested at the G_2-M phase of the cell cycle. Recently, we could show that the checkpoint is ATR and CHK1 dependent (16). Having shown that 293T Lα cells retain intact DNA damage checkpoint pathways, we now examined the response of these cells to MMC, CCNU, melphalan, and psoralen-UVA, agents that form interstrand cross-links (ICLs), to cisplatin, which forms preferentially 1,2-GG and 1,2-AG intrastrand cross-links, to camptothecin and etoposide, representative inhibitors of topoisomerase I and II, respectively, and to IR.

The cytotoxicities of all of the above agents were first determined by MTT assays. The cells were exposed for 1 h to different concentrations of the reagents and their viability was measured 4 or 5 days later (see "Materials and Methods"). With the exception of cisplatin, the response of 293T Lα^+ and 293T Lα^- cells to the reagents was very similar (Fig. 1*B*). The IC_{50}s (Table 1) for the MMR-proficient and -deficient cells were 0.92 ± 0.15 and 0.87 ± 0.13 μM for CCNU, 468.5 ± 4.9 and 488 ± 2.8 nM for melphalan, 38.5 ± 8.3 and 46.6 ± 12.3 ng/ml for MMC, and 63.8 and 62 J/m^2 for psoralen-UVA. In the case of cisplatin, the 293T Lα^+ cells were slightly more sensitive to killing by this drug than 293T Lα^- cells (3.3 ± 0.1 *versus* 6.7 ± 1.4), as seen with other human cell lines (17–19). However, the contribution of the MMR system toward the cytotoxicity of this drug appears to be somewhat smaller than reported in other studies.

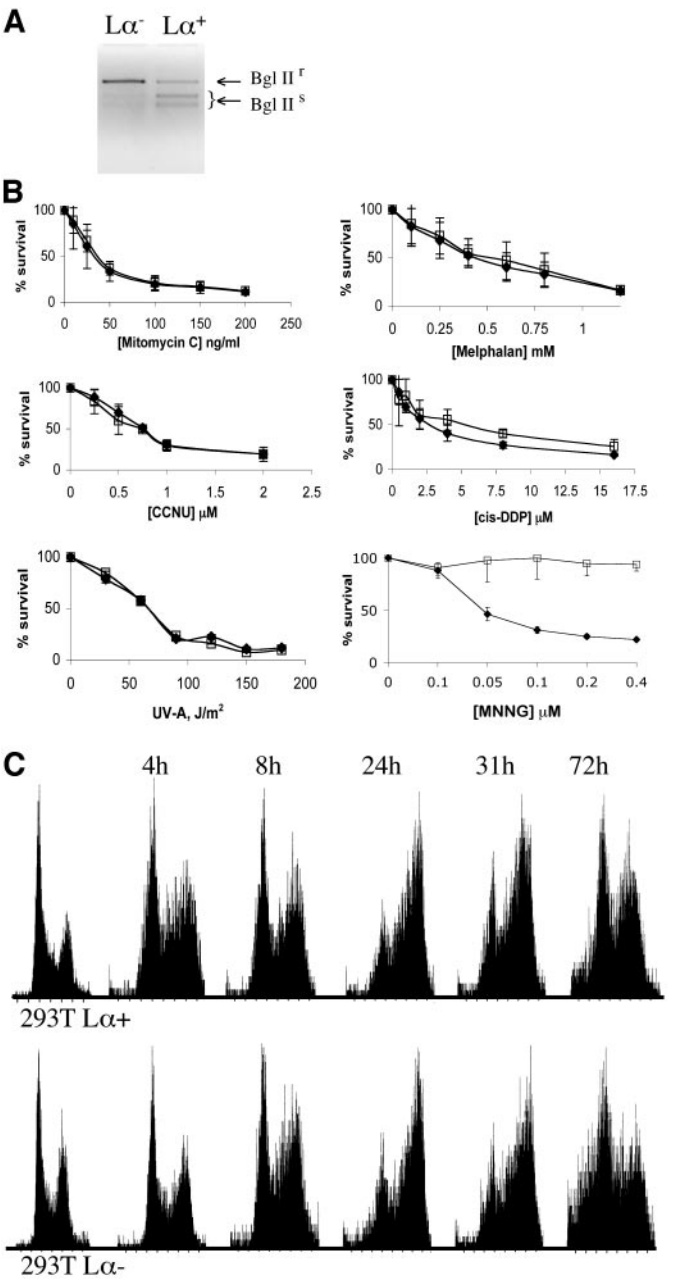

Fig. 1. *A. in vitro* mismatch repair (MMR) assay. The heteroduplex substrate containing a single mismatch (one extrahelical nucleotide) is refractory to cleavage with *Bgl*II (*Bgl*II^r). Repair of the mismatch regenerates the *Bgl*II cleavage site (*Bgl*II^s). The figure shows that the extracts of the 293T Lα^- cells are MMR deficient, whereas those of 293T Lα^+ cells are MMR proficient. See "Materials and Methods" for details. *B*, cytotoxicity induced in MMR-proficient 293T Lα^+ (■) and MMR-deficient 293T Lα^- cells (□) by alkylating agents and cisplatin. Each point represents the mean of three independent experiments with triplicate cultures. *Bars* represent SDs. Cell survival was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assays. *C*, cell cycle distributions of MMR-proficient 293T Lα^+ and MMR-deficient 293T Lα^- cells after treatment (1 h) with an IC_{50} concentration of mitomycin C. Exponentially growing cells (*C*) or mitomycin C-treated cells were harvested 4, 8, 24, 31, and 72 h after treatment and cell cycle analysis was carried out by flow cytometry.

MTT assays detect viable cells in the cultures 4 or 5 days post-treatment but fail to identify arrested cells that may recover later. As the response of our cell pair to CCNU and melphalan was unexpected, inasmuch as MMR-deficient cells were anticipated to be more sensitive to these drugs than MMR-proficient ones (11), we studied the

long-term response of our cells to these drugs also in clonogenic assays. As shown in Table 1, the IC_{50}s determined by both assays were very similar, which implied that the toxicity differences observed in earlier studies were not linked to the MMR status of the cells, but were most likely clone and/or cell type dependent. This hypothesis was additionally confirmed by the finding that the magnitude and duration of a G_2-M cell cycle arrest, induced by treatment with an IC_{50} concentration of MMC, was similar in both 293T Lα^+ and 293T Lα^- cells (Fig. 1*C*).

Treatment of cells with genotoxic agents generally gives rise to a plethora of different modifications in the DNA. Thus, drugs such as CCNU and melphalan generate monoadducts, primarily on guanines, as well as interstrand cross-links (ICLs) and MMC, causes ICLs, intrastrand cross-links, and oxidative damage that leads to base modifications. However, available evidence points to ICLs as the lesions responsible for the cytotoxicity of these drugs, and the same applies to trimethylpsoralen combined with UV light. The molecular mechanism of ICL repair has not been elucidated to date, but it is believed that a subset of proteins involved in nucleotide excision repair and recombination, together with polypeptides linked with *Fanconi anemia*, a clinical syndrome characterized by extreme sensitivity to DNA cross-linking agents, may participate in their detoxification. It is therefore not surprising that the toxicity of drugs generating ICLs may not be affected by the MMR status of the cell.

We next addressed the question whether MMR status modulates cellular response to topo poisons. When the 293T Lα^+ and 293T Lα^- cells were treated with the topo I inhibitor camptothecin or the topo II inhibitor etoposide, MTT assays (Fig. 2) showed no notable differences in sensitivity between the MMR-deficient and -proficient cells (Table 1). Camptothecin and etoposide prevent the re-ligation of single- and double-strand breaks made in supercoiled DNA by topos I and II, respectively. MMR proteins have been reported to participate in recombination-mediated double-strand break repair (19). However, judging by the lack of a differential response of the MMR-proficient and -deficient cells to topo inhibitors, the processing of camptothecin- or etoposide-stabilized breaks does not appear to involve the MMR system.

Table 1 *IC_{50} drug concentrations of mismatch repair-proficient (293T Lα^+) and mismatch repair-deficient (293T Lα^-) cells*

The value marked with an asterisk is taken from Ref. 14. The values represent the mean ± SD of three independent experiments done in triplicate.

Drug	293T Lα^+	293T Lα^-
IC_{50} [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay]		
Chloroethylcyclohexyl nitrosourea (μM)	0.92 ± 0.15	0.87 ± 0.13
Melphalan (nM)	468.5 ± 4.9	488 ± 2.8
Mitomycin C (ng/ml)	38.5 ± 8.3	46.6 ± 12.3
Psoralen-UVA (J/m^2)	63.8	62
Cisplatin (μM)	3.3 ± 0.1	6.7 ± 1.4
Etoposide (μM)	1.17 ± 0.16	1.28 ± 0.15
Camptothecin (nM)	114.4 ± 25.1	115.1 ± 12.3
N-Methyl-*N*′-nitro-*N*-nitrosoguanidine (μM)*	0.05 ± 0.003	6.26 ± 0.5
IC_{50} (clonogenic assay)		
Chloroethylcyclohexyl nitrosourea (μM)	0.819	0.703
Melphalan (nM)	576	678

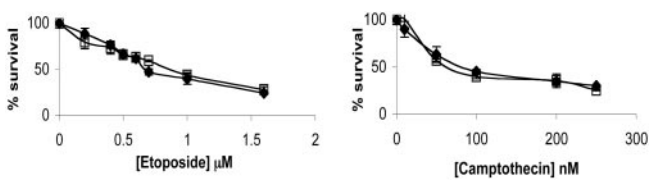

Fig. 2. Cytotoxicity induced in mismatch repair-proficient 293T Lα^+ (■) and mismatch repair-deficient 293T Lα^- cells (□) by the topoisomerase inhibitors camptothecin and etoposide. Each point represents the mean of three independent experiments with triplicate cultures. *Bars* represent SDs. Cell survival was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assays.

Fig. 3. Cytotoxicity induced in mismatch repair-proficient 293T Lα^+ (■) and mismatch repair-deficient 293T Lα^- cells (□) by ionizing radiation. Each point represents the mean of three independent experiments with triplicate cultures. *Bars* represent SDs. Cell survival was determined by clonogenic assays.

IR also generates many different types of modifications in DNA. Base damage represents ~80% of these modifications, but cytotoxicity of IR is ascribed to the production of single-strand breaks and, primarily, double-strand breaks. The repair of these latter lesions is accomplished through homologous recombination or nonhomologous end-joining (21). MMR has been implicated in both processes, but the 293T Lα^+ and 293T Lα^- cells displayed comparable sensitivities to IR (Fig. 3), as described also by others using different matched MMR-positive and -negative cell lines (5, 12). Thus, although MMR-deficient cell lines have been reported by some laboratories to be either slightly more (3, 4) or less (6) resistant to killing by IR than matched MMR-proficient ones, our present results suggest that these differences were unlikely to be linked with the differential MMR status of the cells.

The cells described in this study switch their MMR capability upon Dox addition/withdrawal within only very few cell divisions, and the change of MMR status is not accompanied by noticeable cell death. Thus, where differences in survival to genotoxic stress are observed in this system, they can only be ascribed to the different MMR status of the cells. In this system, inactivation of the MMR system was shown to render human cells slightly more resistant to killing by cisplatin. Coupled with the results of our earlier study, where the 293T Lα^- cells were found to be >100-fold more resistant to killing by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine than 293T Lα^+ cells (14), our data provide clear evidence for the involvement of the MMR system in the cytotoxicity of these two substances and thus fully support the findings of others. In contrast, we failed to detect significant differences in the response of our cells to cross-linking agents, topo poisons, and IR. Thus, the variable response of the other cell systems to these reagents is unlikely to have been associated solely with the MMR status of the cells and must have involved other factors.

The substances investigated in the present study are in frequent use in the therapy of a variety of cancers. The emergence of drug resistance during cancer therapy represents a serious complication, and it is important that its basis is well understood. It is hoped that the isogenic system used in this study will prove useful not only in the elucidation of the molecular mechanisms of drug resistance but also in the identification of substances capable of preferential killing of MMR-deficient cells. Given that a substantial proportion of cancers displays microsatellite instability, these therapeutics are urgently needed.

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