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ABSTRACT

The emergence of resistance to cisplatin is a serious drawback of cancer therapy. To help elucidate the molecular basis of this resistance, we examined matched ovarian cancer cell lines that differ in their DNA mismatch repair (MMR) status and the response to cisplatin. Checkpoint activation by cisplatin was identical in both lines. However, sensitive cells delayed S-phase transition, arrested at G2/M and died by apoptosis. The arrest was characterized by selective disappearance of homologous recombination (HR) proteins, which likely resulted in incomplete repair of the cisplatin adducts. In contrast, resistant cells transiently arrested at G2/M, maintained constant levels of HR proteins and ultimately resumed cell cycle progression. The net contribution of MMR to the cisplatin response was examined using matched semi-isogenic (HCT116+chr3) or strictly isogenic (293T+/-) cell lines. Delayed transition through S-phase in response to cisplatin was also observed in the MMR-proficient HCT116+chr3 cells. Unlike in the ovarian cell lines, however, both HCT116+chr3 and HCT116 permanently arrested at G2/M with an intact complement of HR proteins and died by apoptosis. A similar G2/M arrest was observed in the strictly isogenic 293T+/- cells. This confirmed that although MMR undoubtedly contributes towards the cytotoxicity of cisplatin, it is only one of several pathways that modulate the cellular response to this drug. However, our data highlighted the importance of HR to cisplatin cytotoxicity and suggested that HR status might represent a novel prognostic marker and possibly also a therapeutic target, the inhibition of which would substantially sensitize cells to cisplatin chemotherapy.

INTRODUCTION

Platinum-based drugs are heavy metal complexes containing a central platinum atom, which are frequently used in the treatment of human malignancies. Despite their widespread use, side-effects as well as the acquisition of resistance seriously limit the therapeutic efficacy of platinum drugs in the clinic.1 The cytotoxic activity of cisplatin results from interaction of the highly reactive hydrated form of the drug with DNA, preferentially with the N7 atoms of purine residues.2 Formation of cisplatin-mediated intra- and interstrand crosslinks in DNA causes distortions of the double helix, which inhibit replication,3 transcription4 and translation.5 DNA distortions caused by cisplatin are recognized by a number of proteins and protein complexes,6 such as the MutS protein component of the mismatch repair (MMR) system,7 the high mobility group proteins HMG1 and HMG2,8 histone H1,9 the RNA pol-I binding factor hUBF10 and the TATA-binding protein TBP.11 It is believed that binding of these factors to cisplatin adducts may inhibit or limit the repair of such adducts by the nucleotide excision repair (NER) pathway.6,12

Disruption of the DNA structure by cisplatin-induced interstrand crosslinks completely blocks the progression of the replication fork and lesions of this type have to be processed by homologous recombination (HR).13 On the other hand, 1,2-intrastrand crosslinks generally undergo replication bypass.14 However, since bypass polymerases are error-prone, such lesions are often miscoding and prompt the generation of mismatches.15,16 Recognition of such compound lesions by MutS protein likely explains the involvement of DNA mismatch repair proteins17,18 and may underlie the observed MMR-mediated cisplatin toxicity.19 Hence, cisplatin-resistant cell lines may have a MMR defect, which is most frequently linked with mutations in the hMSH2 or hMLH1 genes, or with the epigenetic silencing of the latter locus.20-23 Experimental evidence, however, showed that reexpression of silenced hMLH1 could rescue the drug sensitivity of the resistant cell lines only to a limited extent,24 with p53-deficiency apparently playing an important role in cisplatin resistance.25 Additional metabolic changes were suggested to contribute to the acquisition of drug resistance.
Among those, an increase in glutathione (GSH) concentration, proportional to the degree of cisplatin resistance, suggested a role for GST in the detoxification processes. Moreover, facilitated excretion of cisplatin and enhanced repair of DNA adducts were proposed to be other possible mechanisms of resistance. Study of the stress signals triggered by cisplatin-induced DNA damage have implicated MAPK (ERK, JNK1/SAPK, p38MAPK) and p53-dependent pathways, as well as the transcription factor ΔNp63α and p73 and the DNA damage-activated tyrosine kinase c-Abl.

In the present study, we studied cell cycle progression and intracellular signaling in a model system consisting of ovarian cancer cell lines that are either sensitive or resistant to cisplatin. In this model, bypass of the cisplatin-induced G_2/M arrest in the resistant cells appears to depend on the maintenance of an intact homologous recombination apparatus, which in turn correlates with more efficient DNA repair. To investigate the net contribution of DNA mismatch repair to cisplatin resistance, we also examined semi- or strictly-isogenic cell lines that are proficient or deficient in the repair of DNA mismatches. We found that the ability to maintain functional homologous recombination machinery was not linked to proficiency in MMR. The implications of our findings for cancer therapy are discussed.

**MATERIALS AND METHODS**

**Cell lines.** The human embryonic kidney (HEK) 293T±Lα cell line was derived from the hMLH1-deficient HEK293T 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., Rockville, MD), supplemented with 10% Tet-System approved fetal bovine serum (Clontech, Palo Alto, CA), 2 mM L-glutamine (Life Technologies), 100 IU/ml penicillin, 100 μg/ml streptomycin (Life Technologies), 100 μg/ml Zeocin (Invitrogen, San Diego, CA), and 300 μg/ml Hygromycin B (Roche Molecular Biochemicals, Basel, Switzerland). Downregulation or induction of hMLH1 (HEK293T-Lα) were obtained by addition or removal of 50 ng/ml Doxycyclin (Dox) (Clontech), respectively, as described.

The human colon cancer cell line HCT116 and its hMLH1-proficient subline HCT116+chr3 were maintained in McCoy's 5A medium (Life Technologies) with 10% fetal calf serum (FCS) (Life Technologies), penicillin and streptomycin. In order to maintain the expression of chromosome 3, 400 μg/mL G418 was added to the medium. The human ovarian carcinoma cell line A2780 and a cisplatin-resistant subline, CP70, were maintained in RPMI 1640 containing L-glutamine and supplemented with 10% FCS, penicillin and streptomycin.

For synchronization experiments, cells were seeded one day before treatment and 2 mM HU was added for 16 h. Cisplatin was added for the last 4 h of incubation before release in complete medium.

**Chemicals and antibodies.** Cisplatin was obtained from Sigma (St. Louis, MO) and dissolved in DMSO, as specified by the manufacturer.

Anti-hMLH1 (554072), anti-hPMS2 (556415) and anti-hRad51 (551922) were from BD Pharmingen (S. Jose, CA); anti-hChk1-pSer345, anti-hChk2-pThr68 and anti-p95-Nav1-pSer143 were obtained from Cell Signaling Technology (Beverly, MA); anti-hChk1 (611152) and anti-hMSH6 (clone 44, G70220) were purchased from BD Transduction Laboratories (S. Jose, CA); anti-hTFFH p89 (sc-19), anti-p53 (Pab 1801), anti-FANCD2 (FL17, sc-20022), anti-BRCA1 (D-9, sc-6954), anti-PCNA (PC10, sc-56) and anti-β-tubulin (D-10) were from Santa Cruz Biotechnology (S. Cruz, CA); anti-p59-Nbs1 (Ab398) was obtained from Abcam; anti-BRCA2 (Ab-1) was from Calbiochem (Darmstadt, Germany); anti-hChk2 (07-126), anti-hCdk1 (60-966), anti-γH2AX-pSer139 and anti-histone H3-pSer10 (06-570) were from Upstate Biotechnology (Charlottesville, VA); anti-RPA-p54 (Ab-3), anti-p21WAF1 (Ab-1, OP64) and anti-hMRE11 (Ab-1, PC388) were from Oncogene (S. Jose, CA); anti-ATM pSer1981 was obtained from Rockland (Gilbertsville, PA) and anti-ATM was kindly provided by Stephen P. Jackson (Wellcome/CRC Institute, Cambridge, UK).

**Western blot analysis.** Cellular proteins were extracted with ice-cold buffer A (50 mM Tris-HCl pH 7.5, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM Na-pyrophosphate, 0.5 mM Na-orthovanadate, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Nonidet P-40). Protein concentration was determined using the Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA). Detection of proteins by Western blot analysis was performed following separation of 50 μg whole cell extracts on SDS-polyacrylamide gels. Proteins were transferred to a Polyvinylidene Fluoride (PVDF) membrane, probed with appropriate antibodies and immune complexes revealed using the ECL system (Amersham-Pharmacia, Upsala, Sweden).

**Immunofluorescence.** Indirect immunofluorescence experiments were performed with cells grown on acid-washed glass coverslips. Fixation was done in ice-cold methanol (20 min at -20°C). Proteins were visualized by overnight incubation at 4°C using anti-γH2AX (1:100), anti-PCNA (1:200) and anti-H3-pS10 (1:100). After washing, the cells were incubated with FITC-conjugated anti-rabbit (1:750, Sigma) and TR-conjugated anti-mouse antibodies (1:200, Abcam) for 1 h at 37°C. Nuclei were counterstained with DAPI (0.1μg/mL, Sigma). Images were captured with an Olympus (IX81) fluorescence microscope.

**MTT assay.** Two thousand cells/well were seeded in 96-well plates one day before treatment. Cells were treated with cisplatin for 4 h, the drug was removed and cells were incubated for five days. Upon addition of the MTT solution (0.5 mg/ml) (Sigma), plates were incubated for 4-5 h at 37°C. One volume of lysis solution (20% SDS, 50% dimethylformamide pH <4.7) was added and the plates were incubated overnight at 37°C. The solubilized formazan was quantified at 570 nm using a Versamax microplate reader (Molecular Devices, Sunnyvale, CA). Optical density values were plotted against the logarithm of cisplatin concentrations and IC_{50} values were calculated from the regression curve.

**Cell cycle analysis.** Cells were harvested at the indicated times, counted, washed with PBS, fixed with 70% ethanol and stored up to one week at 4°C. Cells were then washed with PBS, incubated in PBS containing RNase A (100 μg/ml, Sigma) for 1 h at 37°C, stained with propidium iodide (20 μg/ml, Sigma) and incubated on ice in the dark for 30 min. DNA content was analyzed using a Coulter FC500 Flow Cytometer (Beckman Coulter Inc., Fullerton, CA) and quantification was performed with the software WinMDI 2.8.

**Quantification of S-phase transition.** Cells were seeded in six-well plates at density 6 x 10^3 cells/well and grown in complete medium for 24 h. HU-synchronization and cisplatin treatment were performed as indicated above. One hour before harvesting cells were treated with 10 μM BrdU (Roche, Cell Proliferation ELISA, BrdU colorimetric, Cat. No. 1 647 229) and cells were processed according to the instructions of the manufacturer. BrdU incorporation into nascent DNA was quantitated at 370 nm using 492 nm as reference wavelength in a Versamark microplate reader.
RESULTS AND DISCUSSION

The response of MMR-proficient and -deficient cell lines to cisplatin. A2780 is a human ovarian cancer cell line, from which a cisplatin resistant sub-line (CP70) was derived through exposure to increasing drug concentrations. These matched cell lines are among the few examples of cells of ovarian origin displaying an altered MMR status. A2780 cells are MMR-proficient, whereas CP70 cells are MMR-deficient as a result of hMLH1 gene promoter hypermethylation and consequent lack of MLH1 expression. In our cell viability assays, the CP70 cells were ~10-fold more resistant to killing by cisplatin (Fig. 1A), as reported by others. Clonogenic assays showed that matched pairs of MMR-proficient and -deficient cells, such as the semi-isogenic HCT116±chr3 cells, displayed only ~1.5-fold sensitivity differences to cisplatin. Likewise, in the strictly isogenic HEK 293T-MutLa/La cells, which differ solely in the expression of hMLH1, the sensitivity difference to cisplatin was ~2-fold (293T-MutLa IC₅₀ = 5.3 ± 0.25 μM; 293T-MutLa IC₅₀ = 2.65 ± 0.35 μM; and 15). These results indicated that the larger difference in cisplatin sensitivity observed in the A2780/CP70 system as compared to the isogenic cell lines is likely attributable to traits other than their MMR status, the acquisition of which may have been facilitated by inactivation of the MMR system during clonal selection. Indeed, it has been proposed that genetic instability facilitates the acquisition of drug resistance through the selection of adaptive mutations that occur more copiously in DNA repair-defective cells. In the hope of identifying the pathways responsible for the increased cisplatin resistance of the CP70 cells, we decided to search for differences in the response of the A2780 and the CP70 cells to cisplatin treatment.

Flow cytometric analyses of A2780 and CP70 cells treated with 15 μM cisplatin, which is equivalent to IC₅₀ of the former cells (Fig. 1A),
indicated that the treatment affected progression through the cell cycle in both cell lines (Fig. 1B). The response of the MMR-proficient A2780 cells was characterized by a delayed transition through S-phase (Fig. 1B, 12–24 h) prior to G₂ arrest (Fig. 1B, 48 h) and triggering of apoptosis, as indicated by the presence of a sub-G₁ peak in the flow cytometric profile (Fig. 1B, 72 h). On the other hand, the cisplatin-resistant CP70 cells transited through S-phase with only a slight delay and accumulated at G₂/M (Fig. 1B, 24 h). Moreover, at later time points, the CP70 cells were able to bypass the G₂-block and resume cell cycle progression (Fig. 1B, 48–72 h). To define the point at which the A2780 and CP70 arrested in response to cisplatin, we examined phosphorylation of histone H3, which is indicative of the extent of chromatin condensation in late G₂ and mitosis. Western blot analyses showed that the A2780 cells did not contain phosphorylated histone H3 at any time, thus indicating that the cells were arrested in late S-/early G₂-phase. On the contrary, the CP70 cells displayed a clear signal for the phosphorylated histone, thus confirming that these cells reached the G₂/M transition of the cell cycle (Fig. 1C).

We then examined the pattern of cisplatin-induced signaling in A2780 and CP70 cells. A2780 displayed rapid stabilization of p53 and transcriptional induction of p21<sup>Waf1</sup>, whereas in CP70 the p53 response was delayed and of smaller magnitude (Fig. 1C). More importantly, no p21<sup>Waf1</sup> was detected in CP70 (Fig. 1C). This was likely due to lack of gene induction caused by the nonfunctional p53 protein expressed in CP70 cells,<sup>42</sup> rather than the result of protein degradation consequent to mitotic arrest,<sup>43</sup> since p21<sup>Waf1</sup> could not be detected at any time prior to the G₂/M arrest or following reentry into G₁ (Fig. 1B and C). These data confirmed that the cisplatin-induced p53 stabilization in CP70 cells represents a nonproductive response. Checkpoint pathways were triggered in an identical manner in both ovarian cancer cell lines (Fig. 1D). This response consisted in activation of ATM and phosphorylation of its downstream targets CHK2, BRCA1 and FANCD2. The latter migrated in SDS-PAGE as a doublet, with the slower migrating band likely corresponding to mono-ubiquitylated FANCD2.<sup>44</sup> In this setting, CHK2 displayed long-lasting activation, which is attributable to secondary lesions resulting from stalled replication at sites of damage. On the other hand, CHK1 was only transiently activated. According to their slower progression through S-phase (Fig. 1B), the A2780 cells displayed a slightly more pronounced phosphorylation of NBS1 at 12–24 h, as compared to the CP70 line (Fig. 1D). Despite these similarities, net differences in the signaling pattern of A2780 and CP70 cells could be observed at later times after DNA damage. In G₂-arrested A2780 cells, BRCA1, BRCA2, FANCD2 and RAD51 underwent complete degradation (Fig. 1D, 48 h and 72 h) and the disappearance of these proteins paralleled the activation of an apoptotic response, as indicated by the appearance of the sub-G₁ peak in the flow cytometric profile (Fig. 1B, 48–72 h). In contrast RAD51, BRCA1, BRCA2 and FANCD2 were expressed at all times in CP70 cells. The ability of CP70 cells to resume progression through the cell cycle correlated with a decrease in the size of γH2AX foci (Fig. 1E, 72 h), which may be indicative of either a greater ability to repair DNA damage in these cells or of reduced induction of the DNA repair response consequent to the absence of MMR.
These data showed that the pattern of activation of signaling pathways in the sensitive and resistant cells was very similar during the first 48 h, indicating that the presence of DNA damage was equally well detected and signaled. However, the maintenance of intact homologous recombination machinery likely conferred a selective advantage to the CP70 cells in bypassing the G₂/M arrest.

**The Response to Cisplatin in Isogenic Systems.** Loss of DNA mismatch repair leads to genomic instability through an increased frequency of sporadic mutations in both coding and noncoding regions. To assess the role of MMR proteins in cisplatin-induced signaling more closely, we studied the drug response in matched pairs of MMR-deficient and proficient cell lines. In HCT116+chr3, the lack of MLH1 in the colon cancer cell line HCT116 has been complemented by transfer of an additional copy of chromosome 3, which carries the MLH1 gene. We found that treatment of asynchronous HCT116 and HCT116+chr3 cells with a concentration of cisplatin equivalent to IC₅₀ for the proficient cell line resulted in a similar response in the two cell lines, which was
characterized by a G2 arrest 24 h post-treatment (Fig. 2A). The overall pattern of protein phosphorylation observed in response to cisplatin also seemed to be similar in the two cell lines (Fig. 2B and C). In both cases, the loss of viability that ensued upon prolonged arrest in G2 was paralleled by degradation of DNA repair proteins (Fig. 2B, 72 h). However, closer analysis of the flow cytometric profile possibly revealed a slightly larger S-phase population in the HCT116+chr3 cells at the 24 and 48 h time points (Fig. 2A). In order to examine the cisplatin-induced S-phase delay observed in the mismatch proficient HCT116+chr3 more closely, we analyzed a synchronized population of cells. Cells synchronized at the G1/S boundary with hydroxyurea (HU) were treated with cisplatin. Flow cytometric analysis showed that upon release from the HU-block, the HCT116+chr3 cells were clearly slower in their progression through S-phase, whereas the HCT116 cells accumulated at G2/M (Fig. 3A, 20–24 h + CDDP). Accordingly, phosphorylation of histone H3 was only detectable in HCT116 (Fig. 3B and Supplementary Fig. 1). This confirmed that mismatch repair-proficient cells were blocked in late S-/early G1-phase. The presence of a sub-G1 peak in HCT116+chr3 cells from 20 h of treatment onward indicated that exit from the cell cycle and the triggering of cell death was rapid, likely due to the inability to bypass the block. At later times post-treatment (48–72 h), apoptosis was evident in both cell lines, as indicated by the sub-G1 peak in the flow cytometric profiles (Fig. 3A) and the degradation of cellular proteins such as Cdk1 (Fig. 3B), which is representative of proteins that are expressed throughout the cell cycle. As further support for the fact that the HCT116+chr3 cells displayed a genuine S-phase delay in response to cisplatin, only in these cells could we detect distinct PCNA foci, an indication of ongoing DNA replication, whereas the MMR-deficient HCT116 cells displayed diffuse PCNA staining, typical of cells that have concluded S-phase (Fig. 3C). The S-phase delay was quantified by measuring the incorporation of BrdU into nascent DNA: the data indicated that HU-released HCT116+chr3 cells delayed their transition through S-phase by ~4 h in response to cisplatin (maximal BrdU incorporation at 22 h), as compared to the MMR-deficient HCT116 cells (maximal BrdU incorporation at 18 h) (Supplementary Fig. 2). Foci of γ-H2AX were detected in both cell lines, though they appeared to be larger in the HCT116+chr3 cells (Fig. 3C). In contrast to the A2780/CP70 system, both HCT116 and HCT116+chr3 remained permanently arrested at G2/M (Fig. 3A) and died by apoptosis 48–72 h post-treatment. HR proteins were not preferentially degraded in the MMR-proficient HCT116+chr3 cells, but disappeared in both MMR-proficient and -deficient cells following the kinetics of cell death (Fig. 2B).

Progression of cisplatin-treated 293T-Lα+/− cells, either in MMR-proficient or -deficient state, through the cell cycle (Fig. 4A) and checkpoint activation in these cells (Fig. 4B) were similar to those observed in HCT116+chr3 cells, except that the S-phase delay observed in the MMR-proficient HCT116+chr3 cells was not evident in the MMR-proficient 293T-Lα+ cells. This is likely due to the fact that, among other possible defects, p53 in the latter line has been inactivated by the HPV E6 and the SV40 large-T antigens and could, therefore, not contribute with its S-phase checkpoint function.45

Taken together, the evidence obtained in our study suggests that in an ovarian cancer model (i.e., A2780) as well as in the semi-iso- genic cell line HCT116+chr3, MMR proficiency correlates with the ability of the cells to slow down transition through S-phase in response to cisplatin. However, whether the lack of S-phase delay in CP70 and HCT116 depends only on the MMR status or is additionally contributed to by the acquisition of other genetic alterations, cannot be established at this point. Moreover, the ability to bypass the G2/M checkpoint that accompanied cisplatin-resistance in the CP70 cells examined in our study could not be attributed to the MMR-deficiency alone, since in the semi-iso- genic HCT116 cells and in the strictly isogenic setting of 293T-Lα+/− cells, such bypass did not occur.

The A2780/CP70 cell lines represent a relevant model, since resistance to cisplatin in these cells was induced through a selection process similar to what occurs in patients undergoing chemotherapy. In this system, bypass of cisplatin-induced cell cycle arrest seemed to correlate with the maintenance of intact homologous recombination.

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Figure 4. Cisplatin response of the strictly isogenic MMR-deficient and –proficient 293T-Lα+/− cells. [A] HU-synchronized MMR-proficient 293T-Lα+ and MMR-deficient 293T-Lα− cells were treated with 15 μM cisplatin and cell cycle progression was assessed by flow cytometry at the indicated times. [B] Western blot analyses of total cell extracts derived from the cells shown in [A].
machinery, suggesting that the expression pattern of these proteins could be used in the course of therapy for the diagnosis of emerging drug resistance. Furthermore, our data suggest that future therapeutic protocols might wish to take advantage of the finding that inactivation of the homologous recombination machinery sensitizes cells to the drug. Support for this suggestion can be drawn from knock-out studies of Rad51 paralogs in the B-lymphocyte DT40 model system, where it was shown that defective recombination conferred high sensitivity to cross-linking agents such as mitomycin C or cisplatin. 46 Thus, downregulation of recombination by, for example, siRNA technology, would not only provide a strategy complementary to the proposed inactivation of NER as a means to interfere with the repair of cisplatin-induced damage, 37 but would also counteract the emergence of cisplatin resistance through promoting the massive apoptotic response observed in A2780 cells.

References