Cisplatin and Adriamycin Resistance Are Associated with MutLα and Mismatch Repair Deficiency in an Ovarian Tumor Cell Line

(Received for publication, June 12, 1996)

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In contrast to parental A2780 ovarian tumor cells, extracts of one doxorubicin-resistant and two independent cis-diaminedichloroplatinum(II)-resistant derivatives are defective in strand-specific mismatch repair. The repair defect of the three hypermutable, drug-resistant cell lines is only evident when the strand break that directs the reaction is located 3' to the mismatch, and in each case repair is restored to extracts by addition of purified MutLα heterodimer. As judged by immunological assay, drug resistance is associated with the virtual absence of the MutLα MLH1 subunit and greatly reduced levels of the PMS2 subunit. These findings implicate a functional mismatch repair system in the cytotoxic effects of these antitumor drugs and may have ramifications for their clinical application.

Doxorubicin (adriamycin) and cisplatin1 are widely used as anticancer drugs (1, 2), but their clinical effectiveness is frequently limited by the emergence of drug-resistant tumor cell populations that are often cross-resistant to structurally unrelated drugs (3, 4). Cisplatin reacts with DNA, and the cytotoxic effects of the drug are believed to be due to production of 1,2-intrastrand cross-links between adjacent purines in d(GpG) and d(ApG) sequences (1, 5, 6). The anticancer action of the DNA intercalator doxorubicin has been attributed to production of DNA double strand breaks with protein-linked termini via trapping of topoisomerase II reaction intermediates, but the compound can also participate in redox cycling reactions that produce DNA damage including cross-links (2, 7).

Proteins of the high mobility group family bind to cisplatin 1,2 cross-links (8, 9) and inhibit their in vitro repair by the human excinuclease system (10). However, the relationship between these observations and cisplatin resistance in tumor cells remains unclear. An alternate mechanism for cisplatin resistance is suggested by three observations implicating mismatch repair defects in this phenomenon. Escherichia coli mutants deficient in Dam methylase activity are hypersensitive to the cytotoxic effects of cisplatin, but dam− mutL− double mutants are resistant to the drug (11). Furthermore, microsatellite repeat sequences have been found to be genetically unstable in the majority of cisplatin-resistant isolates derived from an ovarian tumor cell line (12). Microsatellite instability, a phenotype designated RER+ (13), is frequently associated with mismatch repair deficiency (14–16). The human mismatch recognition activity MutSα has been shown to recognize the cisplatin 1,2-(dGpG) cross-link (17). We show here that several RER+ cisplatin and doxorubicin-resistant tumor cell lines are deficient in mismatch repair, with the defect in each case attributable to deficiency of human MutLα (18) activity.

EXPERIMENTAL PROCEDURES

Nuclear Extract Preparation and Mismatch Repair Assays—HeLa, A2780, and drug-resistant cell lines A2780/CP70, A2780/MCP1, and A2780/AD were grown and nuclear extracts prepared as described previously (12, 19). Mismatch repair reactions (19) contained 100 ng of heteroduplex fMR DNA (19–21), 50 μg of nuclear extract, and when indicated 220 ng of near homogeneous MutSα (Fraction III; Ref. 22) or 160 ng of purified MutLα (Fraction VI, about 20% pure; Ref. 18). Incubation was for 15 min at 37°C.

Determination of MLH1, PMS2, and MSH2 Levels in Nuclear Extracts—Nuclear extracts (50 μg) were subjected to electrophoresis through a 7.5% SDS-polyacylamide gel and proteins transferred to a nylon membrane (Biotrans, ICN), which was probed with monoclonal EH12 antibody directed against human MSH2 (a gift from Bert Vogelstein, Johns Hopkins University, Baltimore, MD), monoclonal antibody G168–15 against MLH1 (PharMingen Labs), or monoclonal Ab-1 against PMS2 (Calbiochem). Immune complexes were visualized using an enhanced chemiluminescence reagent (Amersham Corp.).

RESULTS AND DISCUSSION

The ovarian tumor cell line A2780, which does not display microsatellite instability and is thus RER−, has been described previously, as have its RER+ derivatives A2780/CP70 A2780/MCP1, and A2780/AD, which were isolated by in vitro selection for cisplatin resistance in the former two cases and doxorubicin resistance in the latter (12, 23, 24). Clonogenic assays have shown that A2780/CP70 cells are cross-resistant to doxorubicin and that A2780/AD cells are cross-resistant to cisplatin. In view of the association of mismatch repair deficiency with the RER+ phenotype (14–16), we have tested nuclear extracts prepared from these four cell lines for mismatch repair activity using a biochemical assay (20, 21). Repair was scored using heteroduplexes containing a single G-T mispair or a CA- or 5'-heteroduplexes, respectively (20, 21).

Extracts derived from RER+ A2780 cells actively repaired the G-T and CA- insertion/deletion heteroduplexes without regard to location of the strand break 3' or 5' to the mismatch (Fig. 1, white bars). While extracts prepared from all three drug-resistant cell lines supported repair of G-T and CA- or 5'-heteroduplexes, mismatch correction was undetectable with

1 The abbreviations used are: cisplatin, cis-diaminedichloroplatinum(II); MNG, N-methyl-N'-nitro-N-nitrosoguanidine; MNU, N-methyl-N-nitrosourea.

2 P. Vasey and R. Brown, unpublished results.
Cisplatin Resistance and Mismatch Repair Deficiency

Fig. 1. Orientation-dependent mismatch repair deficiency of cisplatin- and doxorubicin-resistant cell lines. Mismatch repair assays (see "Experimental Procedures") utilized heteroduplexes containing a G-T mismatch or a CA dinucleotide insertion/deletion mispair and a single-strand break 181 nucleotides 3′ to the mismatch (3′-heteroduplexes) or 125 nucleotides 5′ to the mispair (5′-heteroduplex) (20–22). White bars, nuclear extract only; black bars, MutSa-supplemented nuclear extract; gray bars, MutLα-supplemented nuclear extract. Repair activity on 3′-heteroduplexes was undetectable in un-supplemented or MutSa-supplemented A2780/CP70, A2780/MCP1, and A2780/AD extracts. The small amount of repair indicated for these extracts in the upper panel corresponds to the detection limit of the assay method.

3′-heteroduplexes. We have previously observed selective 3′-repair deficiency in an RER+ endometrial tumor cell line which falls to produce the MLH1 subunit of the human MutLα activity.3

Mismatch repair deficiency of extracts derived from RER+ cell lines that we have tested to date can be alleviated by addition of purified human MutLα or MutSa (18, 22). MutLα is a heterodimer of the MLH1 and PMS2 gene products (18, 25–27), while MutSa is a heterodimer of the MSH2 and GTBP/p160 products (22, 28–30). Purified MutLα and MutSa were therefore tested for their ability to restore G-T and CA 3′-heteroduplex repair to extracts of the cisplatin-resistant cell lines A2780/CP70 and A2780/MCP1, and the doxorubicin-resistant line A2780/AD. Although MutSa was without effect, MutLα restored 3′-heteroduplex repair in each case (Fig. 1, black or gray bars). Additional evidence for MutSa proficiency and MutLα deficiency in these extracts was confirmed by mixing with extracts derived from repair-deficient MT1 cells (31), which harbor defects in the GTBP subunit of MutSa (22, 32), or from the RER+. H6 colorectal tumor cell line (21), which is deficient in MutLα activity (18) due to mutations in both copies of the MLH1 gene (25). Efficient repair of the G-T 3′-heteroduplex was observed when extract from each of the drug-resistant lines was mixed with that from MT1 cells, whereas repair was not detectable upon mixing with H6 extract (not shown).

Since selective deficiency in 3′-heteroduplex repair is associated with MLH1 null mutations (see above), nuclear extracts of the A2780 cell line and its drug-resistant variants were tested immunologically for presence of the MLH1 subunit of MutLα. While the level of MLH1 in A2780 nuclear extract was similar to that of the HeLa cell control, the MLH1 polypeptide was undetectable in the doxorubicin-resistant and the two cisplatin-resistant derivatives of the A2780 cell line (Fig. 2). Furthermore, the PMS2 gene product, which complexes with MLH1 to form a heterodimer (18), was present in greatly reduced amounts in the drug resistant cell lines. By contrast, the MSH2 subunit of MutSa was present at similar levels in all five cell lines. Levels of the p160/GTBP subunit of the MutSa heterodimer were also similar in this set of cell lines, and absence of the MLH1 gene product was observed in 7 out of 8 additional, independent, cisplatin-resistant isolates derived from the A2780 cell line. Thus, as judged by either biochemical or immunological assay, the drug-resistant derivatives of the A2780 ovarian tumor cell line are deficient in MutLα activity.

We have observed gross reduction in the level of the PMS2 gene product, as judged by immune blot analysis, in other RER+ cell lines that harbor mutations in MLH1.5 H6 colorectal tumor cells are deficient in MLH1 function due to presence of a hemizygous or homozygous UAA chain terminator mutation, but possess apparently normal copies of PMS2 (25). Nevertheless, as in the case of the drug-resistant cell lines described here, extracts of H6 cells contain greatly reduced levels of PMS2. By contrast, extracts of HEC-1-A endometrial carcinoma cells, which lack a wild type PMS2 sequence due to presence of a chain-terminator mutation (33), are devoid of

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3 G.-M. Li and P. Modrich, unpublished results.
4 A. Anthoney and R. Brown, unpublished results.
5 J. Drummond and P. Modrich, unpublished results.
detected PM2 product by monodonal immunological test but contain normal levels of full-length MLH1 polypeptide. These findings suggest that MLH1 may act as a positive regulator of PM2 production or that heterodimer formation with MLH1 may stabilize an otherwise unstable PM2 gene product. Based on these observations, it seems likely that the primary defect in the cisplatin- and doxorubicin-resistant lines examined herein is associated with absence of a functional MLH1 gene product, with the reduction in PM2 level being a secondary consequence of MLH1 deficiency.

The selective defect in 3'-heteroduplex repair observed with drug-resistant cell lines differs from the biochemical phenotype of MLH1-deficient H6 tumor cells (25), which are deficient in repair of both 3'- and 5'-heteroduplexes (21). Since MutLα restores 3'- and 5'-heteroduplex correction to H6 extracts (18) and also alleviates the 3'-heteroduplex repair defect in drug-resistant cells, the basis of these distinct phenotypes is not clear. It is possible that 5'-heteroduplex correction that persists in extracts of the drug-resistant cell lines is supported by the residual levels of PM2 present in these cells, and that failure of H6 cells to support 5'-heteroduplex correction is due to formation of an inactive complex between PM2 and the amino-terminal fragment of the MLH1 polypeptide that is produced from sequences upstream of the UAA chain terminator mutation (25). It is also possible that the drug-resistant ovarian tumor cells studied here contain an activity, which is lacking in H6 colorectal cancer cells, that supports 5'-heteroduplex repair in the absence of MutLα. We cannot distinguish between these and other potential explanations.

Mismatch repair deficiency has been shown previously to confer resistance to the cytotoxic effects of DNA alkylation by N-methyl-N'-nitro-N-nitrosoguanidine (MNNNG) or N-methyl-N-nitrosourea (MNU) (31, 34, 35). The finding that mismatch repair deficiency is also associated with cisplatin and doxorubicin resistance extends these observations to DNA damaging agents that are widely used in the clinical setting. Genetic defects in the GTBP/p160 subunit of MutSα or the MLH1 subunit of MutLα have been documented in MNNNG-resistant cells (22, 25, 32, 36), implicating these proteins in the cellular response to DNA methylation damage. Such findings, coupled with the role of these proteins in correction of DNA metabolic errors, has led to the proposal that these proteins serve as components of a general sensor for genetic damage (31, 37). The results summarized herein provide additional support for this view. It is also important to note in this regard that GTBP/p160 and MLH1 mutant cell lines exhibit a G2 checkpoint defect upon challenge with MNNNG or exposure to the base analog 6-thioguanine (37, 38), and the cisplatin-resistant A2780/CP70 cell line, which is shown here to be deficient in the MLH1 product, displays defective cisplatin-induced G1 and G2 checkpoint responses as compared to parental A2780 cells (39).

The simplest interpretation of these observations is that recognition of certain classes of DNA damage by the mismatch repair system initiates a sequence of events that may lead to cell death, but the molecular mechanism by which this occurs is uncertain. In the case of MNNNG/MNU-induced DNA damage, killing dependent on the mismatch repair system has been postulated to be due to translesion synthesis when the replication fork encounters a miscoding, damaged nucleotide in the template strand (38, 40). The presence of the resulting base pair anomaly in newly replicated DNA provokes the mismatch repair system. Since action of the repair system is restricted to the new DNA strand, the offending lesion persists in the parental template, and newly synthesized DNA is turned over by a futile process of excision and resynthesis, presumably a lethal event. However, alternate mechanisms have not been excluded, and it is conceivable that damage recognition by mismatch repair proteins results in cell death by interface with systems other than the excision pathway involved in processing of DNA biosynthetic errors.

While available evidence indicates that deficiency of GTBP/p160 or MLH1 is sufficient to confer resistance to MNNNG or MNU (22, 25, 32, 36), this may not be the case for cisplatin or doxorubicin resistance. RER+ cisplatin-resistant A2780 cell lines display reduced CIP1 expression and an ionizing radiation-induction G1 checkpoint defect, suggesting attenuated p53 function compared to the wild type p53-expressing parental A2780 line (12). Introduction of a dominant negative mutant p53 gene into A2780 cells leads to increased increased resistance to cisplatin.2 Thus, the presence of a functional p53-dependent apoptosis pathway may be necessary to engage a signal generated by interaction of mismatch repair proteins with cisplatin-induced DNA damage, and it is possible that these two DNA damage response pathways are biochemically linked.

The association of mismatch repair deficiency with cisplatin and doxorubicin resistance described here may have implications for the chemotherapeutic application of these agents in the treatment of RER+ cancers. Conversely, given the propensity of these drugs to select for cells harboring mismatch repair defects and the known predisposition of such cells to tumor development (14–16), it is conceivable that their use may result in secondary cancers of the RER+ class.

Acknowledgments—We thank B. Vogelstein for monoclonal antibody EH12 and E. Korytynski for culturing the cell lines.

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