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Multiple DNA repair mechanisms and alkylator resistance in the human medulloblastoma cell line D-283 Med (4-HCR)

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Abstract *Purpose*: We have previously reported preferential repair of DNA interstrand crosslinks in the 4-hydroperoxycyclophosphamide-resistant human medulloblastoma cell line D-283 Med (4-HCR). We now report further studies that explored the potential mechanisms underlying this repair. *Methods*: Limiting dilution assays and Western, Southern, and Northern blots were used to compare specific differences between D-283 Med (4-HCR) and its parental line D-283 Med. *Results*: D-283 Med (4-HCR) was cross-resistant to melphalan and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), with O^6-alkylguanine-DNA alkyltransferase (AGT) levels of 466 ± 164 fmol/mg protein; AGT levels in the parental line, D-283 Med, were 76 ± 96 fmol/mg. The increase in AGT activity was not a result of gene amplification. Depleting AGT with O^6-benzylguanine partially restored sensitivity to BCNU. Both cell lines were deficient in the human mismatch protein MutLα. ERCC4 mRNA and poly(ADP-ribose) polymerase levels were similar in both cell lines, and ERCC1 mRNA levels were 2- to 2.5-fold lower in D-283 Med (4-HCR). Topoisomerase I levels were 2- to 2.5-fold higher in D-283 Med compared with D-283 Med (4-HCR). *Conclusion*: These results, while illustrating the multiple differences between D-283 Med and D-283 Med (4-HCR), do not explain the enhanced DNA interstrand crosslink repair seen in D-283 Med (4-HCR).

Key words DNA repair · Antineoplastic agents, alkylating · Drug resistance, neoplasm · Tumor cells, cultured · Medulloblastoma

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Introduction

Medulloblastoma, the most common malignant brain tumor of childhood [19], is sensitive to a number of chemotherapeutic agents, particularly alkylating agents such as cyclophosphamide [3, 18]. Unfortunately, resistance to chemotherapy frequently develops, resulting in tumor regrowth and patient death [19].

Resistance to cyclophosphamide is multifactorial, with a broad spectrum of mechanisms including elevated levels of aldehyde dehydrogenase [24, 25] and glutathione [2, 20] and increased activity of glutathione-*S*-transferase [30, 43]. Recently, we have demonstrated that D-283 Med (4-HCR), a medulloblastoma cell line with laboratory-generated resistance to 4-hydroperoxycyclophosphamide (4-HC), displays enhanced repair of phosphoramide mustard-induced DNA interstrand crosslinks [14].

We now report further studies using D-283 Med (4-HCR) which explored a spectrum of DNA repair pathways potentially involved in its resistance to 4-HC.

Materials and methods

Cell lines and culture

The human medulloblastoma cell lines D-283 Med and D-283 Med (4-HCR) were maintained as previously described [14]. HeLa S_3 cells and the human colon carcinoma cell lines SO and RKO were obtained from the Cell Culture Core Facility of the Duke Comprehensive Cancer Center.

Drugs

1,3-bis(2-Chloroethyl)-1-nitrosourea (BCNU) was provided by the Pharmaceutical Research Branch of the National Cancer Institute. Melphalan was provided by Glaxo-Wellcome (Research Triangle Park, N.C.); 4-HC was provided by Dr. Susan Ludeman, and O^6-Benzylguanine (O^6-BG) by Dr. Robert Moschel.

Limiting dilution assays

The cytotoxic activity of 4-HC, BCNU, and melphalan was determined by limiting dilution assay as described previously [20]. Cells exposed to O^6-BG were treated for 10 min in a 100 μ*M* solution prior to alkylator treatment (1 h) and plated in O^6-BG at a dose of 10 μ*M*.

O^6-Alkylguanine-DNA alkyltransferase analysis

Quantitation of O^6-alkylguanine-DNA alkyltransferase (AGT) was performed as previously described [13]. Briefly, alkyltransferase activity was measured as removal of O^6-[^{3}H]methylguanine from a [^{3}H]methylated DNA substrate (5.8 Ci/mmol) after the substrate was incubated with the extract at 37 °C for 30 min. The DNA was precipitated by adding ice-cold perchloric acid (0.25 *N*), and hydrolyzed by the addition of 0.1 *N* HCl at 70 °C for 30 min. The modified bases were separated by reverse-phase high pressure liquid chromatography with 0.05 *M* ammonium formate (pH 4.5) containing 10% methanol. Each assay was performed with a positive control cell line (DAOY cell extract). Protein was determined by the method of Bradford [7], and the results are expressed as femtomoles of O^6-methylguanine released from the DNA substrate per milligram of protein.

Functional assay to determine p53 status

D-283 Med and D-283 Med (4-HCR) were treated with 10 Gy of gamma irradiation or not irradiated. Cells were harvested 6 h after gamma irradiation.

Western analysis of p53

Cells were centrifuged, and the pellet was washed with cold phosphate-buffered saline (PBS). The cell pellets were placed on ice for 30 min in a lysis buffer containing 50 m*M* Tris (pH 8), 5 m*M* EDTA, 150 m*M* NaCl, and 0.5% Nonidet P-40. After 30 min, the pellets were sonicated for 20 s. Cell debris was pelleted and the cell lysate was stored at −20 °C. Cell extracts (100 μg) were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel, and proteins were electroblotted onto a nitrocellulose membrane. The membrane was blocked in 5% nonfat dried milk in PBS/0.1% Tween-20 (PBS-T) for 1 h at room temperature. After two 5-min washes in PBS-T, the membrane was probed with the p53 monoclonal antibody (mAb), DO-1 (Santa Cruz Biotechnology, Santa Cruz, Calif.), diluted to 100 ng/ml in 5% nonfat dried milk in PBS-T. The membrane was incubated for 1 h 30 min at room temperature. After six 5-min washes in PBS-T, the membrane was probed for 1 h at room temperature with the secondary antimouse horseradish peroxidase-conjugated antibody (Amersham, Arlington Heights, Ill.) diluted 1:4000 in 5% nonfat dried milk in PBS-T. After six 5-min washes in PBS-T, the membrane was treated with the enhanced chemiluminescence detection system (Amersham) as recommended by the manufacturer. Proteins were visualized by exposing the membrane to X-ray film (Kodak X-OMAT AR) for 30 s and 5 min.

RNA purification

RNA was obtained from tissue culture cells by the method of Chomczynski and Sacchi [10]. Poly A^+ RNA was isolated by chromatography on oligo(dT)-cellulose (Boehringer Mannheim, Indianapolis, Ind.).

Northern analysis

RNA was separated by electrophoresis, transferred to GeneScreen Plus (DuPont NEN, Boston, Mass.), and hybridized to DNA probes as described previously [27]. The AGT and poly(ADP-ribose) polymerase cDNA clones used in this study were obtained from the American Type Culture Collection. The ERCC1 cDNA clone was from J. Hoeijmakers (Erasmus University), and the ERCC4 cDNA clone was from L. Thompson (Lawrence Livermore National Laboratory). Following a high-stringency wash, the membranes were exposed to storage phosphor screens (Molecular Dynamics, Sunnyvale, Calif.). The exposed screens were read on a Storm 860 PhosphoImager (Molecular Dynamics) and quantified using ImageQuant software. All RNA hybridization signals were normalized by the signal for human γ-actin present in each sample.

Southern analysis

High molecular weight DNA was obtained from tissue culture cells as described previously [6]. Methylation of the *AGT* gene promoter was analyzed by digesting DNA (10 μg) with *Pst*I and *Eag*I, and electrophoresing it in a 1.5% agarose gel in Tris-acetate-EDTA buffer. The DNA was transferred to GeneScreen Plus in alkali and hybridized with the 0.81-kb *Pst*I fragment from pKT200 [23], which had been labeled with ^{32}P by random-primed DNA synthesis (Boehringer Mannheim). Hybridization and washing were performed as described by Pieper et al. [34]. Methylation of the transcribed region of the *AGT* gene was determined by digesting the genomic DNA with *Hpa*II or *Msp*I. After the DNA was electrophoresed on a 0.8% agarose gel and transferred, the DNA was hybridized to ^{32}P-labeled AGT cDNA [34]. The amount of hybridized DNA was determined by either autoradiography or PhosphoImager analysis.

Nuclear extract preparation and mismatch repair assays

HeLa S_3, SO, RKO, D-283 Med, and D-283 Med (4-HCR) were grown and nuclear extracts were prepared as described previously [26]. Each mismatch repair reaction contained 100 ng of heteroduplex f1MR DNA and 50 μg of nuclear extract. Purified MutLα (MonoQ fraction) was used to complement the repair reaction where indicated [29]. Mismatch repair reactions were incubated at 37 °C for 15 min as described previously [33]. For Western analysis, nuclear extract (50 μg) of each cell line was fractionated through a 7.5% sodium dodecyl sulfate-polyacrylamide gel, and proteins were transferred onto a nylon membrane that was probed with antibodies directed against human MSH2, PMS2, and MLH1 (Oncogene, Cambridge, Mass.). Immune complexes were visualized by using an enhanced chemiluminescence reagent (Amersham).

Topoisomerase studies

In Western blot analysis, whole-cell lysates were obtained from log-phase sensitive and resistant cells and immunoblotting of equal cell numbers ($0.5–1.0 \times 10^6$ cells) was performed as previously described [16]. DNA topoisomerase I was detected using the C21 murine mAb [31] kindly provided by Dr. Yung Chi Cheng (Yale University, New Haven, Ct.).

Topoisomerase IIα was localized using 454, our rabbit polyclonal anti-topo IIα antibody [16], and topoisomerase IIβ was detected with JB-1, the rabbit polyclonal anti-topo IIβ antibody recently made in our laboratory against recombinant human topoisomerase IIβ [purified with FPLC (Pharmacia, Piscataway, N.J.)] overexpressed in yeast [5]. Antibodies 454 and JB-1 specifically recognize the α and β isoforms of topoisomerase II, respectively.

Immunofluorescent microscopy

D-283 Med and D-283 Med (4-HCR) log-phase cells (50 000, diluted from the suspension culture with PBS) were spun on slides in a Shandon cytocentrifuge (Pittsburgh, Pa.) for 3 min at 500 rpm and immediately fixed at 4 °C with 4% paraformaldehyde (diluted with PBS from a 16% stock solution obtained from Electron Microscopy Sciences, Fort Washington, Pa.) for 20 min. The cross-linking was stopped by the addition of 1% glycine in PBS, and cells were permeabilized for 2 h by several washes in 1% glycine, 0.5% Triton X-100 in PBS. Rabbit polyclonal antibody 454 against topo IIα (1), mouse mAb C21 against topo I (2), and rabbit polyclonal antibody JB-1 against topo IIβ were all diluted 1:100 with 0.1% Nonidet P-40 and 1% bovine serum albumin in PBS, and were used in combinations of one polyclonal and one mAb in each labeling experiment.

After several washes with PBS over 2 h, the slides were incubated with goat antirabbit IgG labeled with tetraethyl-rhodamine isothiocyanate (for the polyclonal primary antibody) or goat antimouse IgG labeled with fluorescein isothiocyanate (for the primary mAb) (Sigma, St. Louis, Mo.) in a 1:80 proportion for the former and a 1:125 proportion for the latter in 0.1% Nonidet P-40 and 1% bovine serum albumin in PBS for 25 min at room temperature. After several washes in PBS, the slides were dried and covered with coverslips in Vectashield mounting medium of antifade/DAPI (1.1) (Vector Laboratories, Burlingame, Calif.).

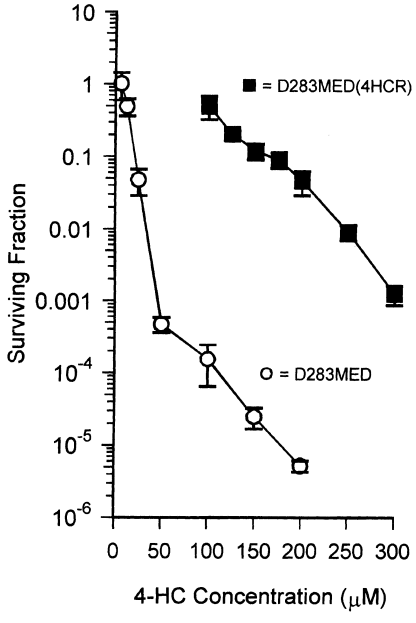

Fig. 1 Survival of D-283 Med and D-283 Med (4-HCR) cells after exposure to various concentrations of 4-HC

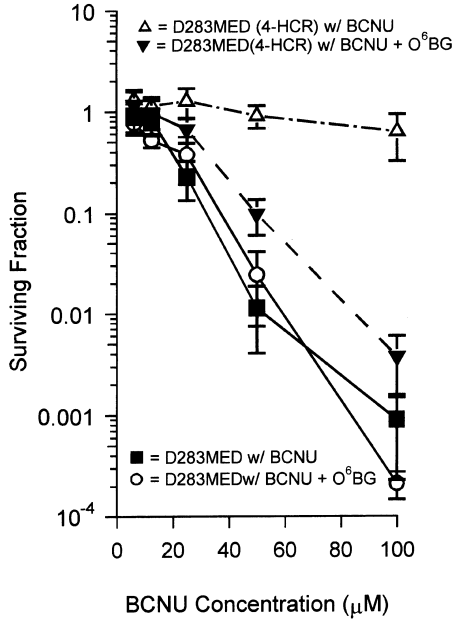

Fig. 2 Survival of D-283 Med and D-283 Med (4-HCR) cells after exposure to various concentrations of BCNU ± O^6-BG ■, D-283 Med; ○, D-283 Med (4-HCR)

Immunofluorescence was observed with a Leitz Orthoplan 2 microscope, and images were captured by a CCD camera with Smart Capture program (Vysis, Downers Grove, Ill.). The intensity of fluorescence in pixels was measured for at least 50 cells in each of two experiments for all three antibodies in both the resistant and parental cell lines.

Results

Limiting dilution assay

The cytotoxicity of 4-HC was markedly less in D-283 Med (4-HCR) than in D-283 Med (Fig. 1). D-283 Med (4-HCR) was cross-resistant to melphalan and BCNU

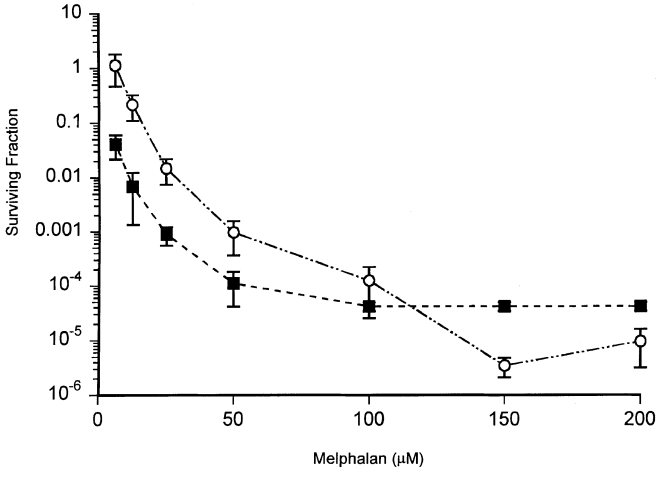

Fig. 3 Survival of D-283 Med and D-283 Med (4-HCR) cells after exposure to various concentrations of melphalan

Fig. 4 AGT transcripts of D-283 Med (*lanes P*) and D-283 Med (4-HCR) (*lanes R*). Poly A^+ RNA (5 μg) from each cell line was analyzed by Northern blot analysis using an AGT cDNA then γ-actin probes

(Figs. 2 and 3). Pretreatment of cells with O^6-BG enhanced BCNU activity against D-283 Med (4-HCR), but not D-283 Med (Fig. 3).

AGT analysis

The AGT levels in D-283 Med and D-283 Med (4-HCR) were 76 ± 96 fmol/mg protein and 466 ± 164 fmol/mg protein, respectively. The amount of AGT enzyme activity measured in cell extracts has been observed to parallel the amount of AGT mRNA in the cells [9]. When the AGT-specific transcripts were determined by Northern analysis of D-283 Med and D-283 Med (4-HCR) RNA, the amount of AGT mRNA in the drug-sensitive cells was below the limit of detection, whereas the 4-HC-resistant cells was detectable (Fig. 4).

Certain regions of cytosine methylation in the *AGT* gene have been found to correlate with enzyme expression. The transcribed region of the gene is generally observed to be more methylated in cells expressing AGT than in cells not expressing the protein [12, 38]. Conversely, there are sequences within the CpG island, located in the promoter of the gene, that are found to be methylated in nonexpressing cells and demethylated in expressing cells [36, 42]. We compared the methylation of both the transcribed region of AGT and the AGT promoter from D-283 Med and D-283 Med (4-HCR). For methylation analysis of the body of the AGT gene, we digested DNA with either *Msp*I or *Hpa*II (Fig. 5A). Comparison of the *Hpa*II digests of D-283 Med and D-283 Med (4-HCR) clearly demonstrated less extensive digestion of the D-283 Med (4-HCR) DNA (Fig. 5A, lanes 3 and 4 versus lanes 7 and 8), indicating that the DNA was more extensively methylated.

We employed *Eag*I digestion to assay methylation within the AGT promoter. The *Eag*I site is 230 bp upstream of the transcription initiation site and is almost completely methylated in Mer^- cell lines [35]. Mer^+ cell lines are partially to totally unmethylated at this site. *Eag*I digestion of the sensitive and resistant cell lines showed that the site in the drug-sensitive cells was completely methylated and in the drug-resistant cells was 30–40% unmethylated (Fig. 5B, lanes 1 and 3). These measurements of cytosine methylation in the *AGT* gene correlate with enzyme activity in the same manner reported for other cell lines. Unlike 3T3 cells treated with 2-chloroethyl-*N*-nitrosourea [40], the increase in AGT

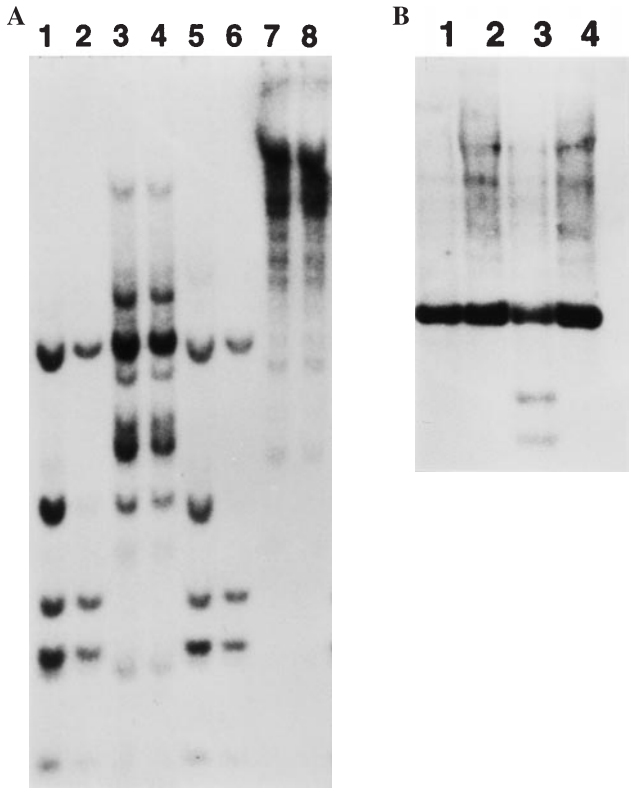

Fig. 5 A Southern blot analysis of the transcribed region of the *AGT* gene. DNA (10 μg) from D-283 Med (*lanes 1–4*) or D-283 Med (4-HCR) (*lanes 5–8*) was digested with 80 units (*lanes 1, 3, 5, and 7*) or 120 units (*lanes 2, 4, 6, and 8*) of *Msp*I (*lanes 1, 2, 5, and 6*) or *Hpa*II (*lanes 3, 4, 7, and 8*). The resulting Southern blot was probed with AGT cDNA uniformly labeled with ^{32}P. **B** DNA (10 μg) from D-283 Med (*lanes 1 and 2*) or D-283 Med (4-HCR) (*lanes 3 and 4*) was digested with 40 units *Pst*I with (*lanes 1 and 3*) or without (*lanes 2 and 4*) 80 units of *Eag*I. The Southern blot was probed with a ^{32}P-labeled 0.81-kb *Pst*I fragment from pKT200 [23]

activity observed in these experiments was apparently not a result of gene amplification (compare Fig. 5A, lanes 1 and 2 with lanes 5 and 6; Fig. 5B, lanes 2 with 4).

p53 studies

Protein p53 levels were significantly higher in D-283 Med compared with its resistant counterpart. Since the p53 mAb recognizes wild-type and mutant p53, a functional assay was required to determine p53 status in these cell lines. Cells with normal *P53* genes exhibit a rapid increase in the levels of wild-type p53 protein after DNA damage, whereas mutant p53 is unaffected. Gamma irradiation is a known to damage DNA, inducing p53 synthesis. D-283 Med and D-283 Med (4-HCR) both demonstrated upregulation of p53 protein levels after gamma irradiation. Therefore, we conclude that both cell lines contain wild-type p53 and that p53 status is not a factor altering AGT levels or influencing resistance of this cell line to 4-HC.

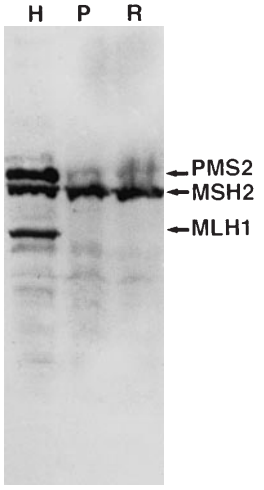

Fig. 6 Western blot analysis of DNA mismatch repair polypeptides in nuclear extracts of D-283 Med and D-283 4-HCR. Nuclear protein extracts from HeLa S_3 (*H*), drug-sensitive (*P*), and drug-resistant lines (*R*) were prepared from exponentially growing cells

DNA mismatch repair studies

Mismatch repair status of both drug-sensitive and -resistant lines was examined by Western blot analysis and DNA mismatch repair assay. As shown in Fig. 6, human MutS protein (MSH2) was present in similar amounts in all three nuclear extracts, whereas human MutLα polypeptides (MLH1 and PMS2) were absent in both drug-sensitive and -resistant lines as compared with HeLa S_3 cells. We also tested the capacity of extracts from each cell line to repair a 3′CA dinucleotide insertion/deletion mispair. Extracts derived from either D283 Med or D283 Med (4-HCR) were defective in the repair of this mispair (Fig. 7). Addition of purified MutLα was able to restore mismatch repair function in extracts from both drug-sensitive and -resistant lines (Fig. 7). These results indicate that mismatch repair function is defective in D-283 Med and D-283 Med (4-HCR) and that the defect is due to the absence of MutLα.

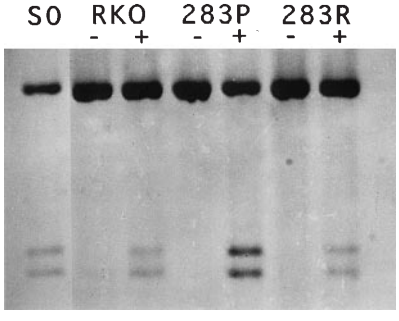

Fig. 7 Restoration of DNA mismatch repair function in D-283 Med and D-283 4-HCR by human MutLα. Nuclear extracts from drug-sensitive (*283P*), drug-resistant (*283R*), SO, and RKO cells were assayed under the standard conditions using a circular 3′CA insertion heteroduplex as substrate. Correction of the heterology was evaluated by cleavage of the DNA with *Bsp*106 and *Xcm*I restriction endonucleases. Repaired DNA yield the two more rapidly migrating restriction fragments [26, 32]. The SO extract served as a positive control; the RKO extract was a negative control that was complemented by the addition of MutLα. Drug-sensitive and -resistant cell extracts were incubated with mispaired substrate DNA in either the presence (+) or absence (−) of MutLα

Expression of ERCC1, ERCC4, and poly(ADP-ribose) polymerase

To determine whether other proteins that play a role in DNA repair may be involved in resistance to 4-HC, we measured the mRNA levels of ERCC1, ERCC4, and poly(ADP-ribose) polymerase in D-283 Med and D-283 Med (4-HCR). As shown in Fig. 8, there was little difference between the two cell lines in their level of ERCC4 or in their level of poly(ADR-ribose) polymerase mRNA. Only 40–50% of the ERCC1 mRNA in the drug-resistant cell line was observed in the drug-resistant cell line.

Topoisomerase studies

Immunoblot analyses for the expression of topoisomerases were performed with log-phase whole-cell lysates obtained from sensitive and resistant cell lines in ten separate experiments for topoisomerase I expression, nine experiments for topoisomerase IIα, and on five separate occasions for topoisomerase IIβ. The cellular content of topoisomerase IIα and IIβ in the resistant cell line was found to be very similar to that of the parental line: 105% ± 13% and 92.4% ± 26.8%, respectively, for the two isozymes. However, Western analysis for topoisomerase I reproducibly demonstrated 2- to 2.5-fold more topoisomerase I in the sensitive cell line.

Immunocytochemical analyses for the three topoisomerase enzymes confirmed a 2.6-fold greater cellular concentration of topoisomerase I in the parental (sensitive) cell line. The differences in the fluorescence intensity for the topoisomerase II isoforms in the two cell

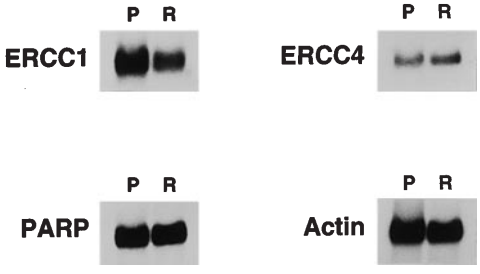

Fig. 8 Analysis of D-283 Med and D-283 Med (4-HCR) RNA. PolyA$^+$ RNA was isolated from the drug-sensitive (*lanes P*) and -resistant (*lanes R*) cell line and hybridized as Northern blots to ^{32}P-labeled cDNA probes from ERCC1, ERCC4, and poly(ADP-ribose) polymerase. After exposure to autoradiography film, each blot was stripped and reprobed with the cDNA for γ-actin

lines were less significant with this method: approximately 1.7-fold more topoisomerase IIα in the sensitive cell line and 1.4-fold more topoisomerase IIβ in the resistant cell line.

Discussion

Alkylator resistance in medulloblastoma, and indeed in all other malignancies studied to date, is multifactorial [14, 20]. We have previously demonstrated enhanced repair of phosphoramide mustard-induced DNA interstrand crosslinks in a 4-HC-resistant medulloblastoma cell line [14].

These studies were designed to evaluate a spectrum of DNA repair pathways potentially mediating or associated with the enhanced repair in D-283 Med (4-HCR). No one mechanism of resistance was considered most relevant; rather a series of potential alterations known to mediate interstrand crosslink repair were studied. Andersson et al. [4] have shown that the nucleotide excision repair genes *ERCC1* and *ERCC4* are involved in the repair of 4-HC-induced DNA damage in alkylator-resistant chronic lymphocytic leukemia cells. However, our study of D-283 Med (4-HCR) and D-283 Med (the parental line) showed that there was no difference in the levels of ERCC4 mRNA in the parental (sensitive) and resistant cell lines, and approximately 40% of the level of ERCC1 mRNA was present in the resistant line compared with the sensitive line.

Although poly(ADP-ribose) polymerase has been associated with response to alkylator-induced damage [8, 11], there was no difference in poly(ADP-ribose) polymerase mRNA levels in the sensitive and resistant cell lines. Analysis of topoisomerase I and II levels (enzymes potentially associated with DNA repair [39]) revealed a marked decrease in topoisomerase I levels in D-283 Med (4-HCR) compared with D-283 Med. These results do not seem to explain the alkylator resistance displayed by D-283 Med (4-HCR) in particular.

DNA mismatch repair deficiency has been noted to confer resistance to methylating agents and cisplatin [1, 15, 22, 28]. We postulated that a deficiency of DNA mismatch repair could be involved in 4-HC resistance in D-283 Med (4-HCR). However, both the parental and the resistant cell lines showed a deficiency of MutLα which, although explaining the previously described poor response to temozolomide ± O^6-BG [21], is not relevant to the 4-HC resistance in D-283 Med (4-HCR).

Cross-resistance to different alkylating agents has been described by other investigators [17, 37, 41]. D-283 Med (4-HCR) is cross-resistant to melphalan and BCNU, which is surprising because 4-HC and melphalan produce disparate N7 guanine crosslinks and BCNU produces N1,3 guanine-cytosine crosslinks after adduct formation at the O^6 position of guanine. Unexpectedly, AGT levels were markedly elevated in D-283 Med (4-HCR), which explained at least partial cross-resistance to BCNU. Depletion of AGT with O^6-BG reduced BCNU resistance, albeit not to parental levels of sensitivity.

These studies leave the precise mechanism of the enhanced repair of DNA interstrand crosslinking in D-283 Med (4-HCR) unexplained. Presumably, alterations in other repair systems such as post-replication repair [32] may be responsible for the DNA interstrand crosslink repair. We are currently refining an assay using a phosphoramide mustard to induce DNA interstrand crosslinking in an oligonucleotide, with a plasmid vector as a means of defining the DNA repair pathways underlying the resistance of D-283 Med (4-HCR).

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